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Impact of annealing on the susceptibility of wheat, potato and pea starches to hydrolysis with pancreatin

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Abstract

Native, and one- and two-step annealed wheat, pea and potato starches were subjected to hydrolysis with pancreatin (1.34 nKat/mg starch, 37 °C, pH 6.0). While annealing increases enzyme resistance for wheat, pea and potato starches in the first (rapid) phase of hydrolysis, it increases the extent of degradation in the second (slower) phase for wheat and pea starches. Annealed potato starches, however, are still more resistant than native potato starch in the second phase of hydrolysis. Environmental scanning electron microscopy shows that enzymic degradation of wheat starch granules does not proceed uniformly throughout the granule population. Pancreatin action does not affect differential scanning calorimetry (DSC) gelatinisation characteristics of all studied native and of annealed potato starches. Although enzymic hydrolysis has no great effect on the DSC gelatinisation behaviour of native starches, partial enzymic solubilisation of the granules enhances the effects of annealing. After 2 and 120 h of solubilisation, DSC thermograms of annealed wheat and pea starches show somewhat broader peaks with lower enthalpies than those of the corresponding unhydrolysed starches. ^{13}C CP/MAS NMR data of extensively (46%) degraded and undegraded native wheat starch granules show no change in double helix content, whereas after 57% solubilisation of one-step annealed wheat starch, a decrease in the proportion of double helices is observed. The ^{13}C CP/MAS NMR signal at 31 ppm increases by a factor 2.0 for 46% solubilised native wheat starch, and by a factor 2.3 for 57% solubilised annealed wheat starch, indicating resistance of amylose–lipid complexes to pancreatin hydrolysis. Dissociation enthalpies, however, are higher than can be predicted from a concentration of complexes. The enthalpy of dissociation of amylose–lipid complexes, after enzymic hydrolysis, increases more for

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annealed than for native wheat starch. All the above suggest that, during annealing, molecular changes occur that have an impact on pancreatin hydrolysis. © 1998 Elsevier Science Ltd

Keywords: Starch; Annealing; Enzymic hydrolysis; Differential scanning calorimetry; ^{13}C CP/MAS NMR; Environmental scanning electron microscopy

1. Introduction

Light and electron microscopy show that starch granules consist of concentric layers. These layers presumably represent growth rings and are alternating regions of high and low density, refractive index, crystallinity and resistance to acid and enzymic hydrolysis [1]. The dense layer of a growth ring consists of alternating crystalline and amorphous lamellae, whereas the low density layer is largely amorphous and contains more water and consequently less starch. The crystallinity of the granules (15–45%) is ascribed to amylopectin, rather than to amylose. The branches of amylopectin form double helices and pack together resulting in formation of the crystalline lamellae, leaving the branch points in the amorphous lamellae. Amylose molecules are believed to be interspersed between amylopectin [2] and most of the amylose is probably located in the low density layers of the growth rings [3].

Granular starches are more resistant towards α-amylolysis than gelatinised starch. Several factors, such as botanical origin of the starch or amylase source, affect the rate and extent of hydrolysis of granular starches. Structural parameters of the granule are also important: crystallinity [4–7], granule size and available specific surface [7–9], amylose to amylopectin ratio [4,8,10], accessibility, porosity, structural inhomogeneities [9] and degree of integrity are amongst those mentioned most frequently. Factors limiting enzymic hydrolysis are diffusion in the granule [6,9,11] and adsorption [9] of the enzyme on the starch molecules. Inhibition of α-amylase by hydrolysis products such as maltose and maltotriose [12] is known to occur [9,11,12].

The kinetics of α-amylolysis are characterised by an initial rapid hydrolysis phase followed by a slower phase [6,7,11,13,14]. The reduction in the hydrolysis rate is probably caused by product inhibition of the α-amylase activity [11]. During bacterial or pancreatic α-amylolysis amorphous parts are thought to be more rapidly degraded than crystalline zones [5,6]: according to Colonna and Buléon [6], the volume of a starch double helix is not compatible with that of the active site of α-amylases. Therefore, hydrolysis of a crystalline starch substrate requires a preliminary disentanglement of chains by the same enzyme [11]. Hydrolysis of wheat [11] and of potato [13] starches with bacterial α-amylase occurs granule by granule. Thus, such enzyme action is not uniform throughout the granule population. This contrasts with acid hydrolysis which occurs throughout the granules with preferential attack on the amorphous parts [15,16]. Bacterial enzymic hydrolysis does not influence the crystallinity and differential scanning calorimetry (DSC) thermograms of starches [11,17–19]. Also, the amylose to amylopectin ratio of corn, wheat and oat starches is not affected by α-amylolysis, even after 50% solubilisation [11,13,17]. However, when residues of fungal or pancreatic α-amylolysis are observed with scanning and transmission electron microscopy, successive internal layers are observed, corresponding to alternating areas with strong and weak susceptibilities [7,20]. Less susceptible regions include crystalline lamellae [7,20]. Extensive hydrolysis of most starches leads to the formation of individual blocklets delineated by simultaneous radial and tangential hydrolysis [5,7].

The detailed mechanism of α-amylolysis depends on starch botanical source. The pancreatic α-amylolysis of wheat starch (A-type) is characterised by the formation of holes and preferential disruption of the core of the granule [5,20]. In a first stage, susceptible zones are pitted. These pits enlarge and numerous canals are formed by endocorrosion towards the center of the granule. Apart from such radial hydrolysis, tangential hydrolysis occurs in the most susceptible layers of the granule. This leads to the observation of a sawtooth pattern at the edges of the radial channels and in a later stage the successive amorphous and crystalline layers in the granule. Potato starch, which is a B-type starch, is very resistant to degradation by pancreatic α-amylase [13,14,21]. Planchot et al. [7] observed an extent of degradation of only 5% with porcine pancreatic α-amylase (1.34 nKat/mg starch). Potato starch granules are predominantly superficially hydrolysed, some endocorrosion occurs at the hilum and the surrounding area [20]. Several explanations have been given for the extreme resistance of potato starches to α-amylolysis. The presence of phosphorus, which is relatively abundant in potato starch (0.1% of which 75% is in the form of glucose

6-phosphate), would form a steric hindrance to the action of α-amylase [22]. According to Ring et al. [8], the large particle size and thus low available specific surface would account for the slow degradation of potato starch. Colonna and Buléon [6] ascribe the low susceptibility of B-type starches to the presence of a great number of crystallites at the surface of the granules. In contrast, for smooth pea starch (a C-type starch), hydrolysis with *Bacillus amyloliquefaciens* α-amylase, extensively eroded surfaces are observed, and for wheat starch, a preferential attack in the most amorphous layers of the granule takes place [23]. The digestibility of C-starches would be intermediate between that of A- and B- starches [10].

Amorphous amylose–lipid complexes in native cereal starch granules [24,25] are fairly resistant to α-amylolysis [18,19,26–28]. Synthetic inclusion complexes of amylose with monoacyl glycerols are hydrolysed more slowly with pancreatic α-amylase than granular wheat starch [28]. Furthermore, the enthalpy of dissociation of amylose–lipid complexes in the starch granules increases after α-amylolysis of wheat starch [11,18,19], indicating an enrichment of these complexes after partial solubilisation of the granules. This was confirmed by measurement of an increased phosphorus content after α-amylolysis [18], since most phosphorus in wheat starches is present in the form of phospholipids. The nitrogen content, on the contrary, is not increased, which indicates that proteins are solubilised together with starch [18].

Annealing of starch is defined as incubation of granular starch in excess water at a temperature above the glass transition but below the gelatinisation temperature. The definition implies that no gelatinisation occurs during the treatment. It has significant effects on starch physicochemical properties [29–35]. The gelatinisation temperature is increased, the gelatinisation temperature range is narrowed and the gelatinisation enthalpy is increased or unchanged [30,31,33]. Also, the starch pasting properties are affected [33,34]. Annealing does not result in changes in the wide angle X-ray diffraction patterns [29,31]. The small angle X-ray diffraction patterns, however, are intensified [35], indicating an increased electron density contrast between crystalline and amorphous regions. No change in repeat distance of crystalline and amorphous lamellae in wheat (105 Å) and potato (99 Å) starches was observed as a result of annealing [35].

The annealing treatment also affects the susceptibility to enzymic hydrolysis. Lorenz and Kulp [36] found annealing to increase the susceptibility of wheat starch to degradation with a fungal α-amylase. More recently, a slightly decreased degree of solubilisation of wheat starch, and also of lentil and potato starches, after 72 h of porcine pancreatic α-amylolysis, was reported [32]. The latter was attributed to increased interactions between starch components during annealing. For annealed barley [17] and oat [32] starches, an increased enzymic susceptibility was found.

The purpose of this work was to further investigate the impact of one- and two-step annealing on enzymic hydrolysis of wheat, pea and potato starches, and to characterise native and annealed starches before and after hydrolysis with pancreatin.

2. Experimental

Materials.—Wheat (Meriwit I) and potato (Meridal G) starches were from Amylum (Aalst, Belgium). Pea starch (Nastar) was from Cosucra (Momalle, Belgium). Pancreatin with α-amylase, lipase and protease activities (porcine stomach mucosa, P1625) and *Streptomyces griseus* protease (EC 3.4.24.31, P5147) were from Sigma Chemical, St. Louis, MO, USA. The Ceralpha kit for measurement of α-amylase activity was from Megazyme, Bray, Ireland.

Enzyme assay.—α-Amylase activity (expressed in nKat) was measured before every experiment, using the Ceralpha-kit [37] with non-reducing-end blocked *p*-nitrophenyl maltoheptaoside as a substrate. α-Glucosidase activity was measured using *p*-nitrophenyl α-D-glucopyranoside as a substrate with the same reaction conditions (1.0 M malic acid buffer pH 6.0, 37 °C) and based on the same principle as the α-amylase activity determination. No significant α-glucosidase activity was detected in the pancreatin extract.

Kinetics of enzymic hydrolysis.—Starch suspensions (3.0 g/100 mL) were incubated at 37 °C with pancreatin (1.34 nKat/mg starch) in a 1.0 M malic acid buffer (pH 6.0), containing 1.0 M NaCl, 40 mM $CaCl_2$ and 0.1% NaN_3, for up to 120 h, with continuous agitation. At specific time intervals, 1.0-mL aliquots were taken after homogenisation of the suspensions. After filtration (0.22 μ), the soluble carbohydrates in the filtrate were measured by the phenol-sulphuric acid method [38]. Every experiment was performed in triplicate. The following parameters were determined: the initial and final hydrolysis rates V_i (initial slope) and V_f (final slope), the easily degradable fraction EDF, calculated as the intercept of the fitted line with slope V_f, and the final hydroly-

sis value FHV, taken as the degree of hydrolysis after 120 h.

Isolation of the insoluble residues after enzymic hydrolysis.—After 2 or 120 h of hydrolysis, residual starches were isolated by filtration (0.22 μ). The residues were washed several times with deionised water, and subsequently suspended in 0.1 M KH_2PO_4 buffer (20.0 mL, pH 7.5). *Streptomyces griseus* protease solution (5.0 mL, 0.5 mg/mL buffer) was added and the suspensions were incubated for 2 h at 37 °C. This protease treatment was included to remove enzymes, possibly adsorbed on the granules. Afterwards, the suspensions were filtered as above, residues were washed with deionised water and dried overnight at room temperature. Moisture contents were determined with a Metrohm 701 KF Titrino (Metrohm, Herisau, Switzerland) by the Karl Fischer method [39,40].

Differential scanning calorimetry (DSC).—DSC experiments were performed with a Seiko DSC-120 (Kawasaki Kanagawa, Japan). Indium and tin were used as standards. Approximately 5 mg of starch were accurately weighed in an aluminium sample pan. Water was added to obtain a dry matter:water ratio of 1:2 (w/w), sample pans were hermetically sealed and heated from 5 to 150 °C (4 °C/min) with an empty pan as reference. The transition temperatures T_o, T_p and T_c are respectively the onset, peak and completion temperatures of the gelatinisation endotherm. T_{am-l} is the dissociation temperature of the amylose–lipid complexes. The enthalpies of gelatinisation (ΔH) and of amylose–lipid complex dissociation (ΔH_{am-l}) were determined by integration using Seiko software. The reported values are means of triplicate measurements.

Annealing procedures.—Starch suspensions (1:2 w/w) were heated for 24 h in a sealed container in a water bath at a constant temperature. The annealing temperatures were chosen as a function of the gelatinisation temperature of the native starches, i.e., 3 to 4% below the gelatinisation peak temperature (in K) as determined by DSC [33]. After a 24 h incubation period, the suspensions were Buchner-filtered and the residues were dried overnight (room temperature, air stream). The resulting starches are further referred to as one-step annealed starches. Two-step annealed starches were prepared by incubating the starch suspensions, after a first period of 24 h at the first annealing temperature, another 24 h at a higher temperature, 3% to 4% below the gelatinisation temperature (in K) of the one-step annealed starches. After this 48 h incubation, the two-step annealed starches were isolated in the same way as the one-step annealed starches. Annealing temperatures for the first and second step were 48 °C and 53 °C, 50 °C and 56 °C, and 50 °C and 55 °C for wheat, pea and potato starches, respectively.

The native starches hydrolysed for 120 h were annealed in DSC pans (dry matter:water ratio 1:2). The sealed pans were incubated in a water bath for 24 h at the same temperatures as for one-step annealing of the unhydrolysed native starches, i.e., 48 °C, 50 °C and 50 °C for hydrolysed wheat, pea and potato starches, respectively. The DSC thermograms were recorded immediately after incubation and cooling down, using the sealed pans.

Environmental scanning electron microscopy (ESEM).—Environmental scanning electron microscopy was performed using an Electro Scan ESEM 3. Samples were fixed to the sample table with double-stick tape and imaged uncoated. A beam accelerating voltage of 12 kV was applied. An environmental secondary electron detector (ESD) was used. Images were taken at ambient temperature and at a sample chamber pressure of 8.0 Torr of water vapor. The working distance (D) from the specimen to the detector ranged from 5.0 to 6.1 mm. The magnification ranged from 1000 to 1500 ×.

^{13}C cross polarization / magic angle spinning (CP / MAS) nuclear magnetic resonance (NMR).—Solid state CP/MAS ^{13}C NMR spectra were obtained at 75.45 MHz on a Bruker AMX-300 instrument with a 4-mm double-bearing probe head. A spinning rate of 10 kHz and spin locking and decoupling fields of about 50 kHz were used. The contact time was 3 ms and a recycle time of 3 s was applied. Other parameters were the following: number of scans, 10,000; spectral width, 62.5 kHz; acquisition time, 35 ms; time domain points, 4 K; transformation size, 8 K; and line broadening, 30 Hz. The spectra are referenced to external Me_4Si.

3. Results

Effect of annealing on hydrolysis kinetics.—Examples of pancreatin hydrolysis kinetics of native, one-step and two-step annealed wheat and potato starches are shown in Figs. 1 and 2. Averages for the parameters of all examined starches are listed in Table 1. As expected, all hydrolysis curves show a two-step pattern: a first rapid phase of solubilisation, and a second slower or plateau phase of solubilisation.

Wheat starches.—Of the three examined native starches, native wheat starch is most susceptible to

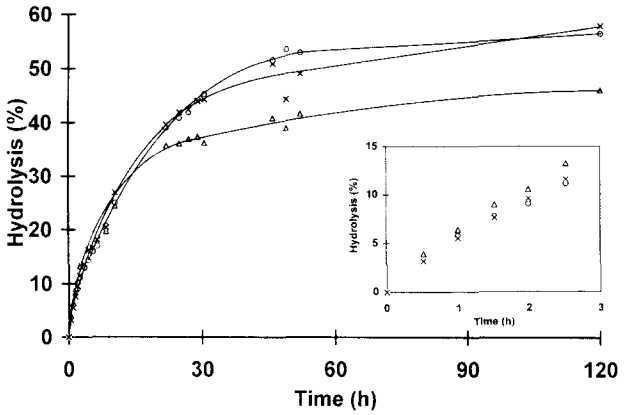

Fig. 1. Pancreatin hydrolysis kinetics (1.34 nKat/mg starch, 37 °C, pH 6.0) of native (△), one-step (○) and two-step (×) annealed wheat starches.

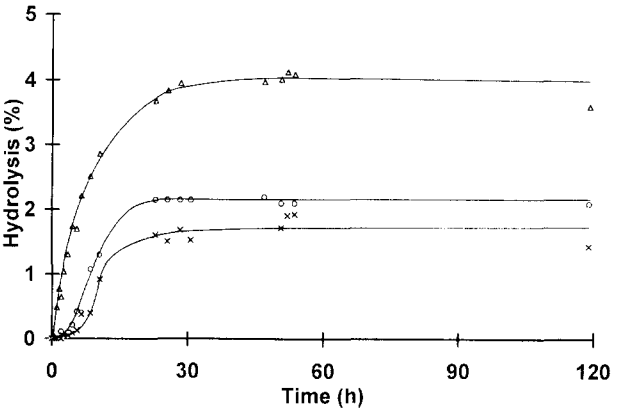

Fig. 2. Pancreatin hydrolysis kinetics (1.34 nKat/mg starch, 37 °C, pH 6.0) of native (△), one-step (○) and two-step (×) annealed potato starches.

enzymic hydrolysis: a final hydrolysis value (FHV) of 54% is obtained (Table 1). One- and two-step annealed wheat starches are even more susceptible to pancreatic α-amylolysis than native wheat starch (Fig. 1). However, this only becomes obvious in the second (slower) phase of hydrolysis (after ±20 h). During the first phase, the annealed starches actually seem to be more resistant than the native one (Fig. 1, inset), but differences in degrees of hydrolysis are very small. The initial (V_i) and final (V_f) rate of hydrolysis are not significantly altered by annealing (Table 1). Both the easily degradable fraction (EDF) and the final hydrolysis value (FHV) increase after one-step annealing, with 10% and 8%, respectively, but do not increase further after a second step of annealing (Table 1). Due to different absolute degrees of solubilisation in the three independent experiments performed for each starch type, standard deviations are large, and no significantly different parameters between native and annealed wheat samples were found. However, for the three independent experiments, the same order in susceptibility for native and annealed wheat starches, as in Fig. 1, was always observed. Thus, although absolute values of the parameters may vary, the order is maintained. Our observations agree with earlier data of Lorenz and Kulp [36] but disagree with results of Hoover and Vasanthan [32], who observed a slightly decreased pancreatic α-amylolysis of annealed wheat starch after 72 h of hydrolysis.

Table 1
Parameters of pancreatin hydrolysis (1.34 nKat/mg, 37 °C, pH 6.0) of wheat, potato and pea starches[a]

	V_i[b] (% h)	V_f[c] (% h)	EDF[d] (%)	FHV[e] (%)
Wheat starches				
native	4.7 (0.3) **A**	0.13 (0.08) **A**	38 (7) **A**	54 (6) **A**
one-step annealed	4.3 (0.1) **A**	0.05 (0.18) **A**	48 (12) **A**	62 (7) **A**
two-step annealed	4.7 (0.3) **A**	0.11 (0.03) **A**	48 (6) **A**	62 (3) **A**
Pea starches				
native	3.5 (0.4) **A**	0.01 (0.03) **A**	35 (4) **A**	37 (2) **A**
one-step annealed	2.6 (0.2) **B**	0.18 (0.05) **B**	31 (3) **A**	53 (5) **B**
two-step annealed	3.5 (0.1) **A**	0.14 (0.04) **B**	34 (6) **A**	50 (6) **B**
Potato starches				
native	0.34 (0.03) **A**	−0.002 (0.003) **A**	4.1 (0.5) **A**	3.8 (0.7) **A**
one-step annealed	0.09 (0.04) **B**	0.005 (0.006) **A**	1.8 (0.3) **B**	2.3 (0.5) **B**
two-step annealed	0.08 (0.06) **B**	0.004 (0.008) **A**	1.4 (0.4) **B**	1.9 (0.8) **B**

[a] Means of triplicate measurements (standard deviations between brackets), different characters indicate significant differences on a 5% level according to the Tukey test.
[b] Initial slope (0–2.5 h for wheat and pea, 0–6.5 h for potato starch).
[c] Final slope (46–120 h for wheat, 48–120 h for pea and 30–120 h for potato starch).
[d] Easily degradable fraction, calculated as intercept of fitted line with slope V_f.
[e] Final hydrolysis value, taken as the degree of hydrolysis after 120 h.

Pea starches.—Native pea starch is less susceptible to enzymic hydrolysis than native wheat starch. The FHV observed after 120 h is 37% (Table 1). The effect of annealing on pea starch is approximately the same as for wheat starch. After one-step annealing, the increased initial resistance to enzymic attack is more pronounced than for wheat starch and a significantly lower V_i is found (Table 1). For one- and two-step annealed pea starches, V_f and FHV are significantly higher, but no differences were found in the EDF (Table 1).

Potato starches.—In agreement with earlier findings [7,13,14,21], native potato starch highly resists enzymic attack. Even after 120 h, only 4% of the starch is solubilised (Table 1). This value agrees well with the 5% degree of hydrolysis reported by Planchot et al. [7] for the same dosage of pancreatic α-amylase (1.34 nKat/mg starch). A number of explanations for this extreme resistance were suggested above. In contrast with observations for wheat and pea starches, annealing even increases the already high resistance to enzymic hydrolysis of potato starch both in the rapid and slow phase of hydrolysis (Fig. 2). V_i strongly decreases after annealing, while V_f is unchanged (Table 1). One- and two-step annealing halve both the EDF and the FHV values of the native starch (Table 1). Enzymic susceptibilities of one- and two-step annealed starches do not differ significantly. The increased enzyme resistance for annealed potato starches agrees with observations made by Hoover and Vasanthan [32].

Microscopic characterisation of hydrolysed wheat starches.—Degrees of hydrolysis of the wheat starch residues, isolated after 2 and 120 h of pancreatin hydrolysis are listed in Table 2. The wheat starch granule population consists of small and large granules. No changes are observed after annealing (Fig. 3a,b). After 2 h of hydrolysis (12–13%), some of the native and one-step annealed granules show some pits, while other granules seem to be unaffected. After 120 h of hydrolysis of native wheat starch, larger holes are observed in most granules, while some granules still have a smooth surface or show only small pits (Fig. 3c). The granular structure is retained after 46% of solubilisation. Fig. 3d shows that granular structure is not retained for all annealed starch granules. It is of note that they are solubilised to a greater extent (57%) than native wheat starch granules after 120 h of hydrolysis. Some granule fragments are observed. In the remaining granular structures, some large holes or small pits are observed. The above suggest that degradation of wheat starches with pancreatin does not proceed uniformly over the granule population. The same phenomenon was already observed by Colonna et al. [11] for bacterial α-amylolysis of wheat starch.

DSC characterisation of annealed starches.—One- and two-step annealing of wheat, pea and potato

Table 2
Thermal characteristics of pancreatin hydrolysed native and annealed wheat starches

Wheat starches	T_o (°C)	T_p (°C)	T_c (°C)	$T_c - T_o$	ΔH (mJ/mg)
0 h hydrolysis					
native	54.4 (0.0)	58.7 (0.1)	63.6 (0.1)	9.2 (0.1)	11.8 (0.3)
one-step annealed	61.6 (0.1)	63.7 (0.2)	66.9 (0.2)	5.3 (0.2)	12.2 (0.4)
two-step annealed	64.2 (0.1)	66.3 (0.1)	69.7 (0.1)	5.5 (0.0)	12.3 (0.1)
2 h hydrolysis[a]					
native (13%)	56.1 (0.2)	60.0 (0.2)	65.7 (0.5)	9.6 (0.4)	12.5 (0.9)
one-step annealed (12%)	62.1 (0.1)	65.3 (0.1)	69.1 (0.2)	7.0 (0.2)	11.5 (0.3)
two-step annealed (12%)	63.5 (0.0)	68.1 (0.2)	72.0 (0.3)	8.5 (0.3)	10.8 (0.2)
references[b]					
native	55.5 (0.1)	58.9 (0.0)	63.5 (0.1)	8.0 (0.2)	12.4 (0.3)
one-step annealed	61.5 (0.1)	63.9 (0.1)	67.3 (0.1)	5.8 (0.1)	11.4 (0.2)
two-step annealed	64.1 (0.1)	66.3 (0.1)	70.0 (0.2)	5.9 (0.2)	11.4 (0.3)
120 h hydrolysis[a]					
native (46%)	56.6 (0.2)	61.1 (0.2)	67.4 (0.6)	10.9 (0.6)	12.0 (0.2)
one-step annealed (57%)	60.7 (0.2)	65.1 (0.1)	69.9 (0.2)	9.2 (0.3)	9.5 (0.3)
two-step annealed (59%)	64.6 (0.9)	66.2 (0.9)	71.2 (1.0)	9.6 (0.3)	8.4 (0.3)

Starch:water = 1:2 w/w; onset (T_o), peak (T_p) and conclusion (T_c) temperatures; gelatinisation ranges ($T_c - T_o$); and gelatinisation enthalpies (ΔH). Standard deviations between brackets.
[a] Degrees of hydrolysis between brackets.
[b] 120 h at 37 °C without enzyme.

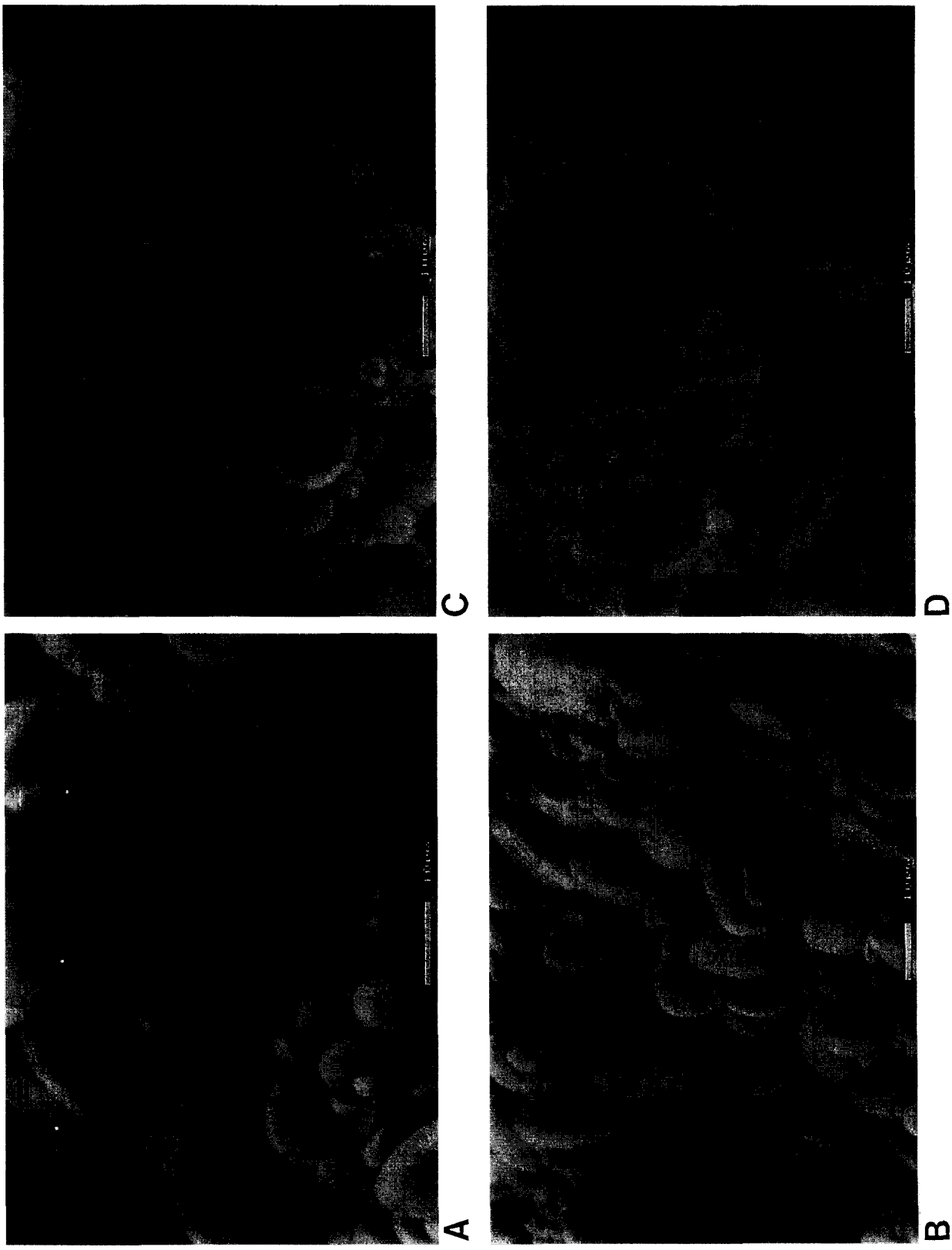

Fig. 3. ESEM images of native (a) and annealed (b) wheat starches, and the respective residues after 120 h of pancreatin hydrolysis (c and d). Images were taken at 12 kV, room temperature and under 8.0 Torr of water vapor pressure. Magnifications and working distances are 1500× and 5.3 mm (a), 1050× and 5.2 mm (b), 1100× and 6.0 mm (c), and 1000× and 6.1 mm (d).

Table 3
Thermal characteristics of pancreatin-hydrolysed native and annealed pea starches

Pea starches	T_o (°C)	T_p (°C)	T_c (°C)	$T_c - T_o$	ΔH (mJ/mg)
0 h hydrolysis					
native	52.9 (0.3)	59.5 (0.1)	68.5 (0.2)	15.6 (0.5)	12.6 (0.8)
one-step annealed	64.6 (0.4)	67.1 (0.2)	70.5 (0.4)	5.9 (0.4)	12.6 (0.6)
two-step annealed	68.1 (0.1)	70.2 (0.1)	73.4 (0.2)	5.4 (0.1)	12.2 (0.3)
2 h hydrolysis[a]					
native (9%)	55.1 (0.1)	61.2 (0.3)	69.5 (0.6)	14.5 (0.6)	12.5 (0.5)
one-step annealed (6%)	64.7 (0.2)	68.3 (0.5)	72.8 (0.7)	8.0 (0.5)	10.2 (0.4)
two-step annealed (8%)	68.5 (0.2)	72.3 (0.4)	77.3 (0.5)	8.8 (0.4)	9.3 (0.2)
references[b]					
native	56.8 (0.0)	60.9 (0.2)	67.0 (0.0)	10.2 (0.0)	14.0 (0.4)
one-step annealed	65.0 (0.0)	67.2 (0.1)	71.3 (0.4)	6.3 (0.4)	13.6 (0.5)
two-step annealed	68.1 (0.1)	70.5 (0.2)	74.2 (0.1)	6.1 (0.1)	12.1 (0.6)
120 h hydrolysis[a]					
native (38%)	57.7 (0.2)	62.4 (0.1)	70.4 (0.4)	12.7 (0.4)	13.3 (0.4)
one-step annealed (58%)	65.1 (0.2)	69.8 (0.1)	76.0 (0.2)	10.9 (0.1)	9.6 (0.4)
two-step annealed (56%)	68.3 (0.2)	73.2 (0.3)	79.0 (0.3)	10.7 (0.1)	5.0 (0.1)

Starch:water = 1:2 w/w; onset (T_o), peak (T_p) and conclusion (T_c) temperatures; gelatinisation ranges ($T_c - T_o$); and gelatinisation enthalpies (ΔH). Standard deviations between brackets.
[a]Degrees of hydrolysis between brackets.
[b]120 h at 37 °C without enzyme.

starches narrow DSC peaks and shift them to a higher temperature (Tables 2–4; 0 h hydrolysis). The largest changes occur after the first step of annealing, but further increases of the peak temperatures and narrowing of the gelatinisation intervals after the second step of annealing are still observed. An increase in peak temperature of up to 10 °C for two-step annealed pea starch is noted, together with a peak narrowing from 13.6 °C to 5.4 °C (Table 3). The gelatinisation enthalpy is not significantly increased

Table 4
Thermal characteristics of pancreatin-hydrolysed native and annealed potato starches

Potato starches	T_o (°C)	T_p (°C)	T_c (°C)	$T_c - T_o$	ΔH (mJ/mg)
0 h hydrolysis					
native	58.0 (0.2)	62.5 (0.1)	70.5 (0.8)	12.5 (0.7)	18.7 (0.5)
one-step annealed	64.7 (0.2)	67.5 (0.1)	72.7 (0.4)	8.0 (0.4)	20.0 (0.5)
two-step annealed	67.3 (0.0)	69.8 (0.1)	74.2 (0.1)	6.9 (0.1)	20.5 (0.8)
2 h hydrolysis[a]					
native (1.4%)	58.2 (0.1)	62.6 (0.1)	70.8 (0.3)	12.5 (0.3)	18.4 (0.4)
one-step annealed (0.5%)	63.1 (0.3)	66.2 (0.3)	73.0 (0.2)	9.9 (0.4)	19.8 (0.4)
two-step annealed (0.5%)	64.5 (0.1)	68.4 (0.2)	74.5 (0.2)	9.2 (0.2)	19.5 (0.3)
references[b]					
native	60.3 (0.1)	64.0 (0.1)	70.7 (0.4)	10.5 (0.3)	19.4 (0.3)
one-step annealed	64.4 (0.1)	67.4 (0.0)	73.3 (0.3)	8.9 (0.3)	19.7 (1.2)
two-step annealed	67.5 (0.2)	70.3 (0.3)	76.0 (0.2)	8.5 (0.1)	18.7 (0.3)
120 h hydrolysis[a]					
native (4.5%)	60.5 (0.2)	64.2 (0.2)	71.2 (0.7)	10.8 (0.5)	19.0 (0.9)
one-step annealed (2.9%)	64.5 (0.1)	67.3 (0.1)	73.1 (0.5)	8.6 (0.4)	19.6 (0.5)
two-step annealed (2.8%)	67.4 (0.1)	70.1 (0.0)	75.4 (0.2)	8.1 (0.2)	19.3 (0.1)

Starch:water = 1:2 w/w; onset (T_o), peak (T_p) and conclusion (T_c) temperatures; gelatinisation ranges ($T_c - T_o$); and gelatinisation enthalpies (ΔH). Standard deviations between brackets.
[a]Degrees of hydrolysis between brackets.
[b]120 h at 37 °C without enzyme.

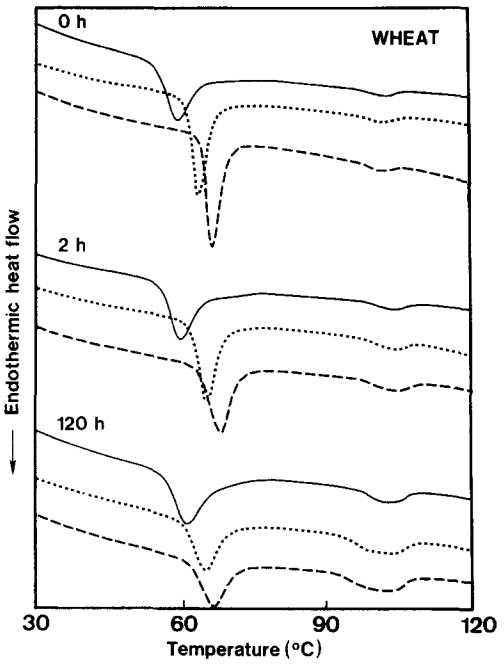

Fig. 4. DSC thermograms of native (————), one-step (· · ·) and two-step (----) annealed wheat starches after 0 h, 2 h and 120 h of pancreatin hydrolysis.

after annealing. For wheat starches, a second endotherm is observed at higher temperature (Fig. 4). This is attributed to dissociation of amylose–lipid complexes [41]. As shown in Table 5 (0 h hydrolysis), annealing does not affect the dissociation temperature or enthalpy of amylose–lipid complexes in wheat starch.

DSC characterisation of residues of native and annealed starches after pancreatin hydrolysis.—Residues of native and annealed wheat, pea and potato starches after 2 and 120 h of hydrolysis with pancreatin were characterized with DSC (Tables 2–5). For all native starches examined, no large changes in gelatinisation behaviour occurred in the course of enzymic hydrolysis, even after solubilisation of more than 50%. Unchanged DSC thermograms for enzymically hydrolysed wheat starches were already observed by Colonna et al. [11].

Wheat starches.—After 2 h of hydrolysis, a small increase in peak temperature (T_p) is noted for native, one-step and two-step annealed wheat starches. For the annealed starches, a slight peak broadening and enthalpy decrease are also observed (Fig. 4, Table 2). After 120 h, the endotherms broaden slightly for all three samples. A further enthalpy decrease is only observed for the annealed samples. Incubation of the wheat starches at 37 °C without enzymes for 120 h does not change the gelatinisation profiles (references in Table 2). This means that no annealing of wheat starches occurs at 37 °C.

As mentioned before, for wheat starches, dissociation of amylose–lipid complexes results in a second endotherm at ca. 102 °C (Fig. 4). A striking observation is the enlargement of this endotherm after 2 h

Table 5
Dissociation temperatures (T_{am-l}) and enthalpies (ΔH) of the amylose–lipid dissociation endotherm of pancreatin hydrolysed native and annealed wheat starches

Wheat starches	T_{am-l}	ΔH_{am-l}	% Hydrolysis	ΔH^a_{calc} (mJ/mg)
0 h hydrolysis				
native	102.4 (0.5)	0.8 (0.1)	0	
one-step annealed	102.3 (0.4)	0.7 (0.1)	0	
two-step annealed	101.7 (0.2)	0.7 (0.1)	0	
2 h hydrolysis				
native	104.4 (0.3)	1.0 (0.2)	13	0.9
one-step annealed	104.1 (0.7)	1.4 (0.1)	12	0.8
two-step annealed	105.0 (0.6)	1.7 (0.2)	12	0.8
references[b]				
native	103.0 (0.4)	0.8 (0.1)	0	
one-step annealed	102.5 (0.2)	0.7 (0.1)	0	
two-step annealed	102.6 (0.4)	0.8 (0.1)	0	
120 h hydrolysis				
native	103.5 (0.3)	2.5 (0.2)	46	1.5
one-step annealed	104.1 (0.7)	4.1 (0.1)	57	1.6
two-step annealed	103.5 (1.1)	4.3 (0.6)	59	1.7

Starch:water = 1:2 w/w; standard deviations between brackets.
[a] $\Delta H_{calc} = \Delta H_{am-l} \times 100$/yield of residue, with yield of residue = 100 − (% hydrolysis).
[b] 120 h at 37 °C without enzyme.

and certainly after 120 h of hydrolysis. This was observed before [11,18,19]. For native wheat starch, the dissociation enthalpy (ΔH_{am-1}) increases with 1.7 mJ/mg after 120 h of hydrolysis, while for annealed wheat starches the increase in ΔH_{am-1} is even more than 3.0 mJ/mg (Table 5). The dissociation temperature (T_{am-1}) does not change much after enzymic hydrolysis. The strongly increased dissociation enthalpy is observed for both native and annealed wheat starches but is even more pronounced for the annealed samples (Table 5).

Pea starches.—After 2 h and 120 h of hydrolysis, the gelatinisation behaviour of pea starches is affected in the same way as for wheat starches (Table 3). After incubation of native pea starch at 37 °C during 120 h without enzymes (references in Table 3), a small annealing effect is observed: increase in the onset temperature (T_o) and narrowing of the endotherm. For the annealed samples, however, no such effect is noted after 120 h at 37 °C without enzymes.

Potato starches.—As for native pea starch, a slight annealing effect is observed after incubation of native potato starch at 37 °C for 120 h without enzymes (Table 4). Again, this is not the case for the annealed potato starches. These observations for pea and potato starches (references Tables 3 and 4) can be explained on the basis of the greater temperature differences between T_o and the incubation temperature of 37 °C. Such differences are 28 °C (28 °C) and 31 °C (30 °C) for one- and two-step annealed pea (potato) starch, respectively, but only 16 °C (21 °C) for native pea (potato) starch. Indeed, the closer the incubation temperature is to T_o, the larger is the effect of annealing [42,43]. After 2 h and 120 h of hydrolysis, no changes are observed in the gelatinisation behaviour of the three potato samples (Table 4).

^{13}C CP/MAS NMR of wheat starches before and after pancreatin hydrolysis.—Solid state high resolution ^{13}C NMR measures order in the starch granule on a molecular and not on a crystalline level. Several signals (81–83 ppm, 94–98 ppm, 102–105 ppm) are characteristic for non-double-helical material, while the signal at 98–102 ppm is characteristic for starch double helices [44]. By comparison of the relative intensities of these signals for different samples, it is thus possible to compare the relative double-helix contents in starch samples.

Comparison of ^{13}C CP/MAS NMR spectra of native and one-step annealed wheat starches does not reveal any differences (Fig. 5a,b), indicating no change in double-helix content as a result of annealing of wheat starch. After 120 h of enzymic hydrolysis of native wheat starch, the relative intensities of the double helix and non-double-helix material signals are also unchanged (Fig. 5c). For one-step annealed wheat starch, however, 120 h of enzymic hydrolysis affects the proportion of these two structural components. Indeed, from Fig. 5d it is clear that the proportion of non-ordered material (signals at 81–83 ppm and 102–105 ppm) has increased.

The small signal at 31 ppm (Fig. 6) is attributed to carbons of fatty acids (free fatty acids or fatty acids in monoacyl glycerols) that are immobilized in amylose–lipid complexes [24,25]. One-step annealing of wheat starch does not affect this signal (Fig. 6a,b), indicating that the amylose–lipid complexes are not affected by annealing of wheat starch. After 46% (120 h) enzymic solubilisation of native wheat starch, the signal intensity at 31 ppm (relative to the total signal intensity between 50 and 110 ppm) doubles (Fig. 6c), while after 57% (120 h) hydrolysis of one-step annealed wheat starch, the same signal has increased by a factor 2.3 (Fig. 6d).

Annealing of 120h-hydrolysed native starches.—Since the DSC gelatinisation behaviour of native starches before and after 120 h of enzymic hydrolysis is practically the same, native starches isolated after 120 h of hydrolysis were annealed in order to find out whether these residues responded to the annealing treatment in the same way as the unhydrolysed native starches. As shown in Table 6, the 46% hydrolysed wheat, 38% hydrolysed pea and 4.5% hydrolysed

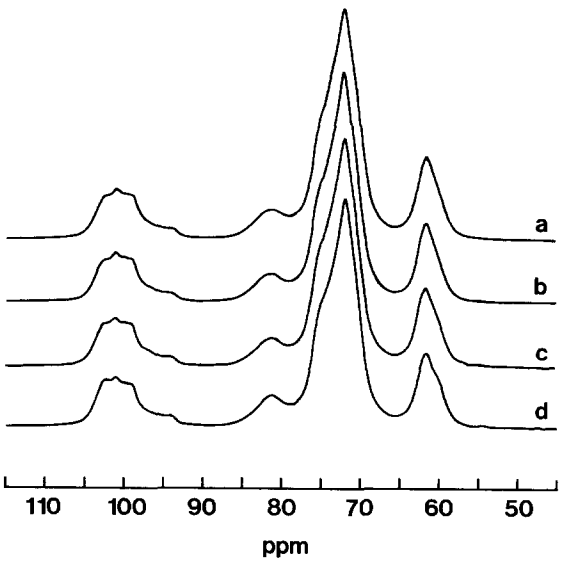

Fig. 5. ^{13}C CP/MAS NMR data of native (a) and annealed (b) wheat starch, and of native (c) and annealed (d) wheat starch after 120 h of pancreatin hydrolysis.

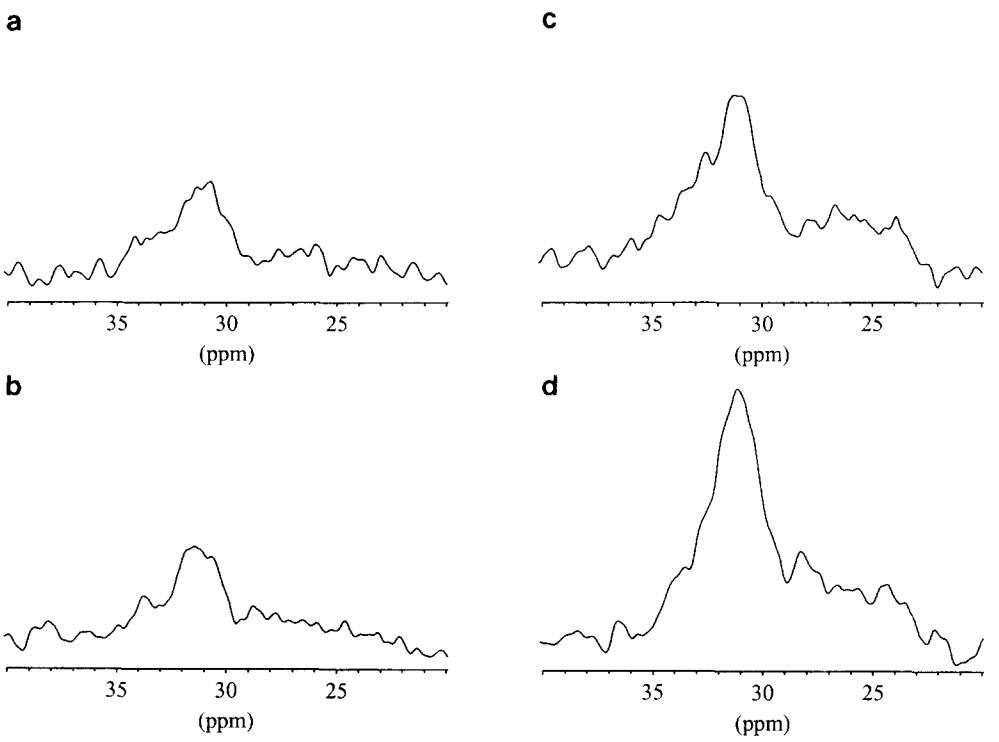

Fig. 6. The ^{13}C CP/MAS NMR signal at 31 ppm for native (a) and annealed (b) wheat starch, and native (c) and annealed (d) wheat starch after 120 h of pancreatin hydrolysis.

potato starches are still susceptible to annealing. They even seem to be annealed to a greater extent than the respective unhydrolysed starches: for hydrolysed starches that were annealed, onset, peak and completion temperatures are higher than for the respective unhydrolysed annealed starches (Table 6). For potato starch, the gelatinisation range is smaller, while for the other starches the range is somewhat larger.

Annealing of the 46% solubilised wheat starch has no effect on the dissociation temperature or enthalpy

Table 6
Thermal characteristics of native wheat, pea and potato starches, annealed[a] after 120 h of pancreatin hydrolysis

	T_o (°C)	T_p (°C)	T_c (°C)	$T_c - T_o$	ΔH (mJ/mg)
Wheat starches					
annealed[b]	61.6 (0.1)	63.7 (0.2)	66.9 (0.2)	5.3 (0.2)	12.2 (0.4)
hydrolysed + annealed	64.9 (0.1)	67.3 (0.1)	71.0 (0.2)	6.1 (0.1)	11.1 (0.2)
annealed + hydrolysed[b]	60.7 (0.2)	65.1 (0.1)	69.9 (0.2)	9.2 (0.3)	9.5 (0.3)
Pea starches					
annealed[b]	64.6 (0.4)	67.1 (0.2)	70.5 (0.4)	5.9 (0.4)	12.6 (0.6)
hydrolysed + annealed	66.3 (0.1)	68.8 (0.0)	73.0 (0.0)	6.7 (0.1)	13.8 (0.5)
annealed + hydrolysed[b]	65.1 (0.2)	69.8 (0.1)	76.0 (0.2)	10.9 (0.1)	9.6 (0.4)
Potato starches					
annealed[b]	64.7 (0.2)	67.5 (0.1)	72.7 (0.4)	8.0 (0.4)	20.0 (0.5)
hydrolysed + annealed	67.2 (0.1)	69.5 (0.2)	74.5 (0.1)	7.2 (0.1)	19.3 (0.4)
annealed + hydrolysed[b]	64.5 (0.1)	67.3 (0.1)	73.1 (0.5)	8.6 (0.4)	19.6 (0.5)

Starch:water = 1:2 w/w; onset (T_o), peak (T_p) and conclusion (T_c) temperatures; gelatinisation ranges ($T_c - T_o$); and gelatinisation enthalpies (ΔH). Standard deviations between brackets.
[a]At 48 °C, 50 °C and 50 °C for hydrolysed wheat, pea and potato starches respectively.
[b]The DSC parameters for one-step annealed starches and for residues of one-step annealed starches after 120 h of hydrolysis (Tables 2–4) are given for comparison.

of the amylose–lipid complexes (103.5 °C and 2.5 mJ/mg, Table 5). This means that even after partial hydrolysis of the granular structure, no rearrangements of the amylose–lipid complexes during annealing that would lead to a different dissociation endotherm take place.

Comparison of the annealing treatment after 120 h of hydrolysis with the treatment of 120 h of hydrolysis after one-step annealing clearly shows that peaks are narrower and/or shifted to a higher temperature in the former than in the latter case (Table 6). Thus, the order in which the two treatments (annealing and enzymic hydrolysis) are applied, is important.

4. Discussion

Hydrolysis kinetics.—Despite the lack of visible changes in granule surface structure (Fig. 3a,b), crystallinity [29,31] or double-helix content (Fig. 5a,b) as a result of annealing, it influences the susceptibility of starches to pancreatin hydrolysis. The impact of annealing on enzymic susceptibility depends on starch botanical origin and/or the crystal type (Figs. 1 and 2). In the first rapid phase of hydrolysis, annealing increases the enzyme resistance of wheat, pea and potato starches. However, in the second slower phase, annealed wheat and pea starches are hydrolysed to a larger extent than the respective native starches, while annealed potato starches are still more resistant to pancreatic α-amylolysis than native potato starch.

Characterisation of pancreatin hydrolysed native starches.—The unchanged gelatinisation behaviour of native starches after enzymic hydrolysis (Fig. 4, Tables 2–4) is in clear contrast with changes after acid hydrolysis of native starch ([45]; unpublished data). In the latter case, broad undefined endotherms that are shifted to higher temperatures are observed even after only 15% of solubilisation. It is generally believed that acid hydrolysis occurs uniformly throughout the granule population, with preferential attack on the amorphous parts in the granule [15,16]. The broad undefined endotherms are then attributed to the disappearance of the cooperative melting of the granules and reflect the inherent inhomogeneity of the remaining crystalline parts [46]. The practically unchanged gelatinisation behaviour of enzymically hydrolysed starches suggests a different hydrolysis mechanism for enzymes than for hydrogen ions. According to Colonna et al. [11], the unchanged DSC thermograms after enzymic hydrolysis can be explained by a hydrolysis mechanism involving granule after granule breakdown, thus without alteration of the overall composition of the total granule population. With electron microscopy, it is indeed observed (Fig. 3c) that pancreatin hydrolysis does not proceed uniformly throughout the wheat starch granule population. However, quite a large fraction of the granules seems to be attacked after 120 h of hydrolysis, which does not agree well with the unchanged DSC profiles. With ^{13}C CP/MAS NMR it was shown that the proportion of double helices and non double helices in wheat starch is unchanged after 46% of hydrolysis (Fig. 5), indicating no preferential attack on one of these components.

Although enzymic hydrolysis has no great effect on the DSC gelatinisation behaviour of native starches, partial solubilisation of the granules by enzymes facilitates annealing (Table 6).

Characterisation of pancreatin-hydrolysed annealed wheat and pea starches.—From Fig. 4 and Tables 2 and 3 it is clear that, although changes after enzymic hydrolysis are rather small compared to changes as a result of acid hydrolysis, the DSC gelatinisation behaviour of one- and two-step annealed wheat and pea starches is affected to a greater extent than for the respective native starches. At 120 h, this can be partly explained by the higher degree of hydrolysis of the annealed starches. However, after only 2 h, the degree of hydrolysis for annealed starches is approximately the same or less than for the native samples, and still, the gelatinisation endotherms of the annealed starches are more affected than for the native one. More specifically, peak width and area are especially affected, while peak position is much less altered. Increased peak width of annealed starches after enzymic hydrolysis may imply a partial destruction of some effects of annealing, such as crystallite perfection [43,47] and/or altered interactions between the crystallites and the amorphous parts [30,31], as a result of the enzymic treatment. However, whatever annealing associated phenomenon is responsible for the increased gelatinisation temperature (T_p), this phenomenon is not destroyed after prolonged enzymic action, since T_p is practically unaffected. If gelatinisation enthalpy reflects mainly the loss of order of double helices on a molecular scale [48], the enthalpy decrease for the annealed starches after hydrolysis may indicate an increased susceptibility of double helical structures in the granule towards enzyme action as a result of annealing. This is supported by NMR evidence for wheat starches. Indeed, the proportion of double helices was observed to decrease after 120 h of hydrol-

ysis of one-step annealed wheat starch (Fig. 5b,d), while this was not observed for hydrolysed native wheat starch (Fig. 5a,c). After 120 h of hydrolysis of annealed wheat starches, no intact granules are observed, in contrast with native starches, and some granule fragments are present together with pitted granules (Fig. 3d).

The above suggest that, during annealing, molecular and/or structural changes occur that influence enzyme action on starch granules.

Amylose–lipid complexes in wheat starches.—Amylose–lipid complexes occur in amorphous form in wheat starch granules [24,25]. Because amylose–lipid complexes are quite resistant towards enzymic hydrolysis [18,19,26–28], one can expect an increase of the DSC dissociation enthalpy as a result of concentration of complexes (Fig. 4, Table 5). When predicting maximum enthalpy values after hydrolysis by assuming even 100% resistance of the complexes and taking into account the yields of residues after 2 h and 120 h of incubation with pancreatin, the observed enthalpies are still higher than the predicted values (ΔH_{calc} in Table 5). This effect is more pronounced for the annealed wheat starches than for the native starch, and implies that the increased ΔH_{am-1} after enzymic hydrolysis can not be explained solely by concentration of the amylose–lipid complexes. A possible additional explanation is based on the assumption that, apart from the existing amylose–lipid complexes in the wheat starch granule, free lipids would also be present inside the granule or at the granule surface. As a result of partial enzymic hydrolysis, amylose chains would, in this view, become more mobile and complex some free lipids (during the hydrolysis or during gelatinisation of the residue in the DSC). The greater amount of complexes would then lead to a higher dissociation enthalpy. According to Morrison et al. [24] and Morrison [49], all wheat starch lipids (mainly lysophospholipids) are already complexed in the native granule, but it is not excluded that there are some free lipids (mainly free fatty acids) present at the surface of the granules.

^{13}C CP/MAS NMR data (Fig. 6) show that the proportion of immobilised lipids in the wheat starch granules increases, after 120 h of hydrolysis, with a factor 2.0 for native wheat starch, and a factor 2.3 for one-step annealed wheat starch. In fact, these factors are the values one can expect assuming even 100% resistance of the complexes and considering the yields of residues after 120 h of hydrolysis (54 and 43% for native and one-step annealed wheat starches respectively). This implies that no extra amylose–lipid complexes are formed during enzymic hydrolysis. Nevertheless, it remains possible that extra complexes are formed during the DSC-run. However, a sixfold increase in the amount of complexes (as would be deducted from the sixfold ΔH_{am-1} increase after 120 h of hydrolysis of one-step annealed wheat starch) seems not very likely. Indeed, the content of adsorbed free fatty acid is well below that of lysophospholipid (84–202 mg/g and 780–1189 mg/g respectively [49]). In that case, one can only assume that besides the formation of extra complexes as a result of enzymic hydrolysis, the environment of the complexes changes leading to different interactions and a different dissociation enthalpy.

Characterisation of pancreatin-hydrolysed annealed potato starches.—No changes are observed in the gelatinisation behaviour of native, one-step and two-step annealed potato starches after 2 h and 120 h of enzymic hydrolysis (Table 4). This seems logical when taking into account the final degrees of hydrolysis of only 4.5, 2.9, and 2.8% for native, one-step and two-step annealed potato starches, respectively. However, when approximately the same extents of hydrolysis are reached in acid hydrolysis of potato starches, gelatinisation endotherms are affected to a much greater extent and differences in gelatinisation behaviour between native and annealed starches almost disappear (unpublished data). This again points to differences between acid and enzymic hydrolysis mechanisms.

5. Conclusions

The impact of annealing on kinetics of pancreatin hydrolysis depends on starch botanical origin and/or crystal type. No large changes in gelatinisation behaviour were observed for native wheat, pea and potato starches after 2 h or 120 h of hydrolysis. For hydrolysed native wheat starch we found an unchanged granule surface structure as well as an unchanged proportion of double helices. The unchanged gelatinisation behaviour of native and annealed potato starches after enzymic action is probably related to their extremely low degrees of hydrolysis. The characteristics of enzymically hydrolysed annealed wheat and pea starches are affected to a greater extent than for the respective native starches. DSC and ^{13}C CP/MAS NMR evidence indicate that annealing increases the susceptibility of double-helical structures in the wheat starch granule towards pancreatin hydro-

lysis. Amylose–lipid complexes in wheat starches are shown to be resistant to enzymic hydrolysis. No extra complexes were formed during hydrolysis, but they may be formed during the DSC run. As a result of enzymic hydrolysis, the environment of the complexes probably changes, leading to different interactions and a higher dissociation enthalpy. The increase in dissociation enthalpy of the complexes is more pronounced for the annealed wheat samples than for the native one. All the observed differences between pancreatin hydrolysis of native and annealed starches suggest molecular and/or structural changes to occur within the granule during annealing.

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