

Enzyme-Resistant Starch. III. X-Ray Diffraction of Autoclaved Amylo maize VII Starch and Enzyme-Resistant Starch Residues¹

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ABSTRACT

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X-ray diffraction was used to follow the development of crystallinity in amylo maize VII starch samples after repeated cycles of autoclaving and cooling. Changes in crystallinity were revealed by the development of reflections at 0.529 and 0.398 nm. Reduced intensities of a peak at 0.449 nm, which likely reflected the V pattern of complexed amylose, suggested that amylose-lipid complexes melted out during repeated autoclaving and cooling. Diffractograms of enzyme-resistant starch (RS) residues, isolated from amylo maize starch samples by treatment with Termamyl and amyloglucosidase, displayed single peaks at 1.662 and broad diffraction lines at 0.537 and 0.398 nm. The patterns suggested that chain fragments, packed in a B-type crystalline structure with a slightly enlarged

crystal lattice, contribute to formation of RS from amylo maize starch. Structures resistant to enzymatic hydrolysis, as assessed by X-ray and differential scanning calorimetry measurements, differed in crystallinity and resistance to thermal dissociation depending on the treatment of amylo maize starch. Differences in association between the chain fragments, presumably originating from amylose and varying proportions of interspersed, less-ordered segments, were assumed to account for these findings. Conditions applied for preparation of RS, such as type of amyolytic enzyme used in the isolation procedure, also affected thermal and crystallographic properties of RS.

Enzyme-resistant starch (RS) has been shown to be a product of starch retrogradation (Berry 1986, Englyst and Macfarlane 1986, Ring et al 1988). Miles et al (1985b) proposed a concept of starch retrogradation encompassing two processes: a short-term development of the gel structure that is governed by the crystallization of amylose and a long-term process that is due to retrogradation of amylopectin. The formation of RS structures is considered to be linked to interchain associations of the amylose fraction (Matsukura et al 1983; Englyst and Macfarlane 1986; Berry et al 1988; Ring et al 1988; Russell et al 1989; Siljeström

et al 1989; Sievert and Pomeranz 1989, 1990). The process of amylose association, which is affected by factors such as temperature, amylose concentration, and molecular size (Ring 1987, Gidley 1989, Kitamura and Kuge 1989) has been studied by several physicochemical techniques. X-ray diffraction studies on cooled amylose gels and amylose precipitated from aqueous solutions indicate that retrogradation of amylose includes crystallite formation (Kitamura et al 1984; Miles 1984, 1985a, 1985b; l'Anson et al 1988; Gidley 1989). The X-ray patterns recorded were identified as B-type. Also, X-ray diffractograms of RS from autoclaved wheat starch were described as B-type patterns (Berry et al 1988, Russell et al 1989, Siljeström et al 1989).

Gel-permeation chromatography of acid-resistant retrograded amylose fragments revealed that amylose association involves linear chain segments with a polymerization degree of 30-50 (Jane and Robyt 1984, Ring et al 1987, Mestres et al 1988). Similar observations were made by Russell et al (1989) and Siljeström

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et al (1989) for RS isolated from autoclaved wheat and maize starch. Those RS preparations showed a distribution of low molecular weight glucans with a peak polymerization degree of about 60.

Differential scanning calorimetry (DSC) thermograms of amylose preparations (Eberstein et al 1980; Biliaderis et al 1985; Ring et al 1987, 1988); aged mixtures of amylo maize starch and water (Russell 1987); and RS isolated from bread (Sievert et al 1989), cooked pasta (Sievert et al 1988), and autoclaved amylo maize, wheat, pea, and potato starch (Sievert and Pomeranz 1990) yielded endothermic transitions at $\sim 160^\circ\text{C}$, which apparently were due to melting of (re)crystallized amylose.

We recently reported (Sievert and Pomeranz 1989) that repeated autoclaving and cooling of amylo maize VII starch induced increasing melting enthalpies of this transition in the starch samples and respective RS fractions. The objective of the present work was to gain further information, mainly by X-ray diffraction, on the structural characteristics of RS from amylo maize starch.

MATERIALS AND METHODS

The amylo maize VII starch we used was from American Maize Products Co., Hammond, IN. The technique of RS formation by repeated autoclaving-cooling cycles was described elsewhere (Sievert and Pomeranz 1989). The enzymatic-gravimetric procedure for determination and isolation of RS included the use of Termamyl L-120, a heat-stable bacterial α -amylase (Novo Laboratories, Inc., Danbury, CT), and amyloglucosidase from *Aspergillus niger* (No. A-3042, Sigma Chemical Co., St. Louis, MO). The procedure, which followed in principle the pattern of the AOAC method for determination of dietary fiber (AOAC 1985), was described previously (Sievert and Pomeranz 1989). RS was considered to be the insoluble residue remaining after enzymatic incubation of the amylo maize starch samples and expressed as percentage (db) of the starch material used. For comparative digestion studies, hydrolysis of the starch samples was performed with α -amylase from porcine pancreas (A-3176, Sigma Chemical Co., St. Louis, MO). A starch sample (0.5 g) was incubated with pancreatic α -amylase (10 units per milligram of starch) in 50 ml of phosphate buffer (pH 6.9) for 16 hr at 37°C . Unless stated otherwise, RS samples were dried under vacuum. The dried residues were ground to a fine powder to pass through a screen with $150\text{-}\mu\text{m}$ openings. Moisture was determined by AACC method 44-15A (AACC 1983).

Differential Scanning Calorimetry

DSC measurements were carried out as described by Sievert and Pomeranz (1989).

Wide-Angle X-Ray Diffraction

X-ray powder diffraction was performed with dried and hydrated starch samples. Hydration of freeze- and vacuum-dried samples was done in a chamber maintained at 4°C and 95 rh for at least 20 hr.

Starch samples were densely packed in an aluminum frame. X-ray diffraction patterns of the specimens were recorded on a Siemens D 500 diffractometer (Madison, WI) operating at 35 kV, 30 mA with CuK_α radiation ($\lambda = 0.154\text{ nm}$). Diffractograms were obtained from $4^\circ 2\theta$ to $30^\circ 2\theta$ with a step size of $0.05^\circ 2\theta$, counting 4 sec on each step. Measurements of each sample, performed in duplicate, were essentially identical. X-ray diffraction data are reported by interplanar d-spacing values expressed in nanometers. Development of crystallinity was evaluated with respect to the integrated normalized intensities of diffraction peaks and sharpness of the patterns. Integrated normalized intensities were calculated on the basis of the number of counts recorded by the scintillation counter.

RESULTS AND DISCUSSION

X-Ray Diffraction of Amylo maize VII Starch Samples

X-ray diffractograms obtained from vacuum-dried native ($\sim 3\%$ moisture) and amylo maize ($\sim 4\text{--}5\%$ moisture) starch samples that

were freeze-dried after being autoclaved and cooled repeatedly for up to 20 cycles, are illustrated in Figure 1. Amylo maize starch after the first autoclaving-cooling cycle showed less intense peaks at 1.675, 0.616, and 0.449 nm than those of the native starch. As the sample was taken through several autoclaving-cooling cycles, the peak at 0.529 nm developed, and a peak at 0.398 nm emerged. At the same time, the 0.449-nm peak decreased in intensity. These observations indicated that repeated autoclave treatment caused a change in the crystalline structure of the starch.

Hydration is known to influence X-ray patterns (Cleven et al 1978, Nara et al 1978, Wild and Blanshard 1986, Buleon et al 1987), and a certain amount of water is necessary to maintain structural ordering as detected by X-ray diffraction. Figure 2 shows that hydration brought out more characteristics of the profiles but did not result in a change in patterns. The pattern of native amylo maize VII starch ($\sim 12\%$ moisture) can be assigned to a combination of B- and V-type crystalline structures: The unique peak at 1.575, the strong peak at 0.519, and the doublet at 0.398–0.372 nm are typical diffraction lines for a B-type form. The peak at 0.449 nm can be interpreted as a V-form due to amylose-lipid complexes (Zobel 1988). It is not unambiguously established, however, that the V-form exists in native starch granules (Morrison 1988 and references cited therein). Hydration of treated amylo maize starch samples from 5 to 9% moisture did not significantly alter the profiles. At higher moisture levels ($\sim 18\%$), the patterns became sharper and more pronounced (Fig. 2).

Concomitantly, with increasing moisture content of the treated samples, an increase in the intensity of the peak at 1.651 nm was noted. With regard to native starch, the opposite effect was observed. The intensity of this reflection increased upon dehydration (compare 0 cycles in Figs. 1 and 2). These findings agree with other crystallographic data (Wu and Sarko 1978, Wild and

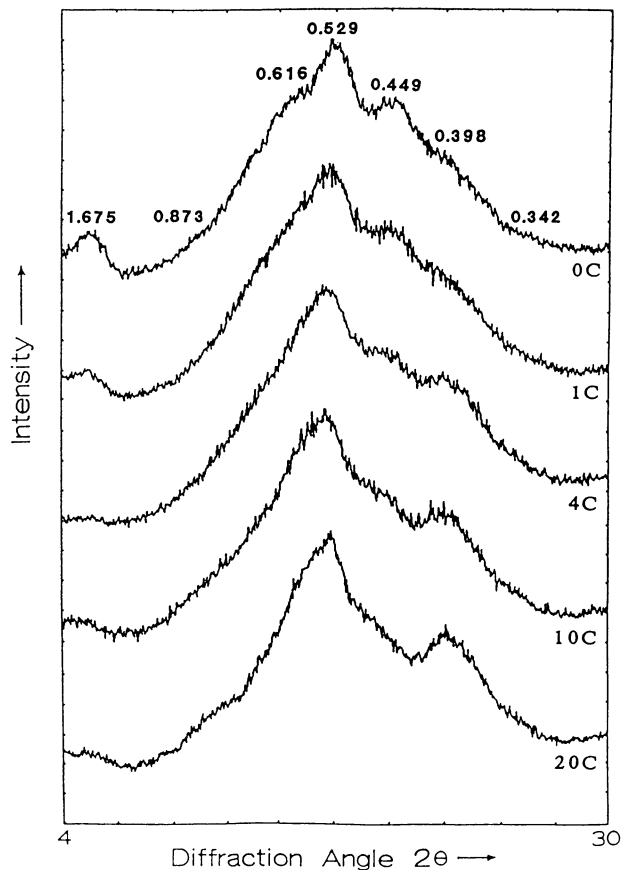


Fig. 1. Effects of repeated autoclaving-cooling cycles (C) on X-ray diffraction patterns of amylo maize VII starch. Vacuum-dried native starch (3% moisture) is shown at 0C, and freeze-dried starch (4–5% moisture) after 1, 4, 10, and 20C. Numbers above peaks indicate interplanar d-spacings in nanometers.

Blanshard 1986, Buleon et al 1987, Zobel 1988), showing that the intensity of the 1.6-nm diffraction line, which reflects packing of double helices, is strongly influenced by hydration.

X-ray profiles of hydrated samples confirmed that a structural transformation of amylo maize starch took place during repeated heating and cooling. The diffraction lines at 0.529, 0.835, and 0.398 nm developed; the reflections at 0.619 and 0.372 nm seemed to disappear; and the 0.449-nm peak decreased in intensity as structures responsible for the latter apparently did not fully recover on cooling. If we accept that the 0.449-nm reflection arose from amylose-lipid complexes, then the reduced intensities of this peak could be interpreted as amylose-lipid crystallites being melted out during repeated autoclave treatment. This is in agreement with results obtained from DSC measurements of the starch samples (results not shown). As we increased the number of autoclaving-cooling cycles, we observed a decrease in melting enthalpy of the transition at about 105°C that corresponds to dissociation of amylose-lipid complexes.

Generally, however, the development of the X-ray profiles indicated that crystallization increased as a result of repeated autoclaving and cooling (Fig. 2). The altered crystalline form of treated amylo maize starch might be due to increasing association of amylose chains, as suggested by DSC measurements of the starch samples (Sievert and Pomeranz 1989). Repeated autoclaving and cooling of amylo maize starch was accompanied by an increase in melting enthalpies of the endothermic transition at 155°C, which can be attributed to melting of (re)crystallized amylose.

Mechanisms of amylose association have been reported to include formation of double helices (Sarko and Wu 1978, Jane and Robyt 1984, Gidley 1989). Gidley (1989) proposed a model involving rigid, double-helical segments interconnected by more

mobile, amorphous single chains. The ordered packing of double helices in amylose gels and amylose aggregations was shown to give B-type patterns (Gidley 1989). The X-ray profiles of autoclaved amylo maize starch samples examined in our study can be interpreted as diffuse or poor B-patterns, showing broad reflections at 0.398 nm that cover the region of the unresolved doublet (0.40–0.37 nm). Apart from the broadening of the peaks, the reflections at 0.529 and 1.651 nm differed slightly but significantly in d-spacing values from their apparent counterparts in native starch (0.519 and 1.575 nm, respectively, Fig. 2). This might be due to the imperfect nature of the crystallites, which could lead to a broadening and overlapping of the 0.616- and 0.519-nm peaks, thereby resulting in the reflection at 0.529 nm. Since the 0.519-nm reflection is a third-order reflection of the inner spacing at 1.575 nm (H. F. Zobel, *personal communication*), the shift of the latter to a higher d-spacing value (1.651 nm, Fig. 2) would be consistent with this hypothesis. Hence, it could be concluded that a B-type crystalline structure with slightly enlarged lattice constants is present in autoclaved amylo maize starch.

X-Ray Diffraction of RS Residues

Diffraction patterns of vacuum-dried RS residues (4–6% moisture) isolated from samples of native and amylo maize starch treated with Termamyl and amyloglucosidase were characterized by weak diffraction lines at 1.662 nm and broad peaks at 0.537 and 0.398 nm (Fig. 3). Hydration of vacuum-dried RS from about 4–6 to 13% moisture resulted in increased intensities of the 1.662-, 0.817-, and 0.398-nm peaks (Fig. 4). Compared with treated amylo maize starch samples (Fig. 2), RS residues gave less sharp diffraction lines in the center of the diffractograms at 0.537 nm and more intense 0.398-nm peaks. Loss of V-type structures in RS residues could be regarded as hydrolysis of less resistant amylose-lipid

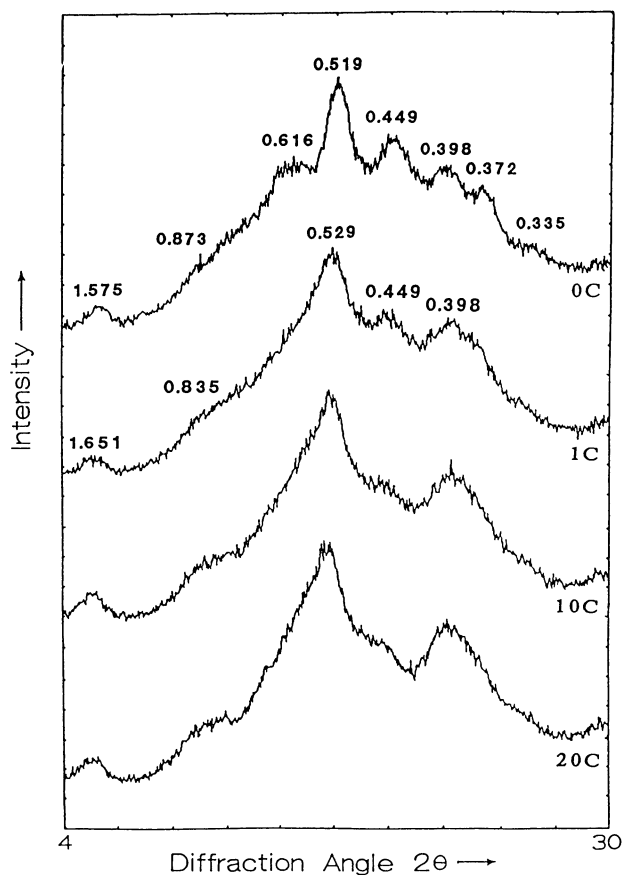


Fig. 2. Effects of repeated autoclaving-cooling cycles (C) on X-ray diffraction patterns of amylo maize VII starch. Native starch (~12% moisture) is shown at 0C, and freeze-dried and hydrated starch (~18% moisture) after 1, 10, and 20C. Numbers above peaks indicate interplanar d-spacings in nanometers.

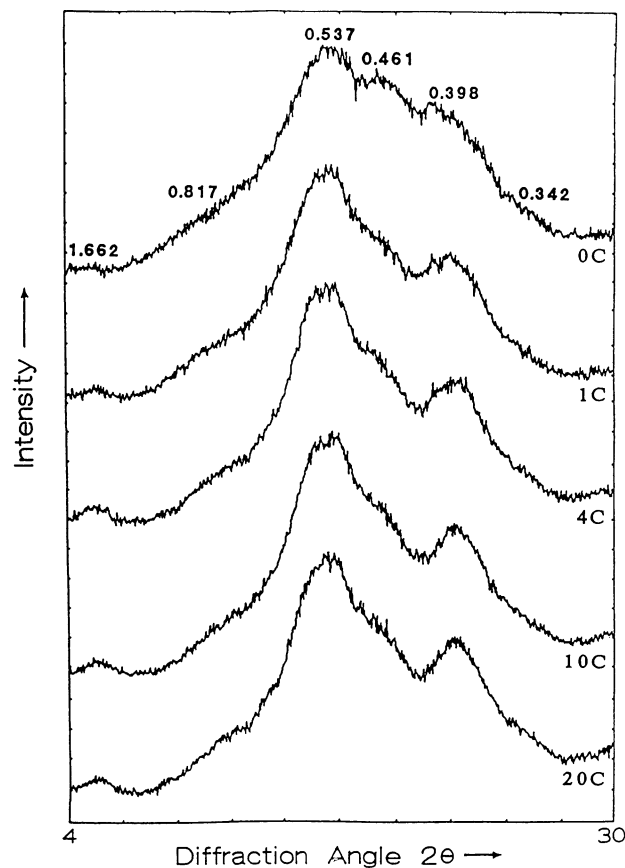


Fig. 3. Effects of repeated autoclaving-cooling cycles (C) on X-ray diffraction patterns of vacuum-dried enzyme-resistant starch (RS) (4–6% moisture) from amylo maize VII starch. RS from native starch is shown at 0C, and from starch after 1, 4, 10, and 20C. Numbers above peaks indicate interplanar d-spacings in nanometers.

complexes since these crystalline structures were found to be accessible to enzymatic action (Jane and Robyt 1984).

As discussed with regard to treated amylo maize starch, RS residues showed the presence of poor B-patterns. This is in agreement with findings of Berry et al (1988) and Russell et al (1989), who observed poor B-patterns of RS from autoclaved wheat starch. The appearance of broad diffraction lines strongly suggested that smaller and/or less perfect crystallites were present in RS than, for example, in native starch, where the sharp, well-resolved pattern (Fig. 2, 0 cycles) reflected a higher degree of crystallite perfection.

Appearance of the 0.537-nm reflection in RS residues (Figs. 3 and 4) appeared to be in accord with the hypothesis that the small and/or imperfect nature of the crystallites accounted for a broadening and overlapping of the 0.616- and 0.519-nm peaks since the enzymatic treatment can be considered as an isolation and concentration of retrograded, ordered amylose fragments present in the starch samples (Sievert and Pomeranz 1989, 1990).

We suggested earlier (on the basis of increasing RS contents and increasing melting enthalpies of the 155°C endotherm of isolated RS residues) that RS structures comprise recrystallized amylose chains that develop upon repeated autoclaving and cooling (Sievert and Pomeranz 1989). Expressed on the basis of equal amounts of RS measured by DSC, Figure 5 illustrates the increase in enthalpy with increasing RS content which, in turn, was induced by repeated cycles of autoclaving and cooling. Development of the endotherm of RS residues appeared to follow a two-stage process, including a rapid increase followed by a much slower rate of development (vacuum-dried RS) or a leveling-off (oven-dried RS). The plateau regions likely reflect a maximum of amylose chain associations in ordered fragments in RS residues that was reached under the conditions applied (e.g., amylose concentration, temperature, time).

Effects of Different Enzyme Preparations

To compare the effects of different enzyme preparations on thermal and crystallographic properties of RS, pancreatic α -amylase instead of Termamyl-amyloglucosidase was used in the isolation procedure. After one autoclaving-cooling cycle, the amount of RS increased to 39.2 from 21.3%; the melting enthalpy of the 155°C endotherm in the RS residue, however, decreased to 9.9 from 21.2 J/g.

Jane and Robyt (1984) described the action of different α -amylases on retrograded amylose. According to them, all amylases preferably hydrolyze the amorphous structures, leaving the double-helical, crystalline regions intact. Based on their findings, it would appear that pancreatic α -amylase was less effective in hydrolyzing disordered chain segments adjacent to the crystalline ordered regions and thus resulted in higher amounts of RS. In contrast, the combined action of heat (100°C) and heat-stable α -amylase, including a subsequent treatment with amyloglucosidase, apparently removed more of the disordered regions and thereby provided lower amounts of RS, with a relatively higher proportion of ordered amylose fragments, as assessed by the higher melting enthalpy of the 155°C endotherm.

Compared with the X-ray pattern of RS from Termamyl-amyloglucosidase incubation, however, the pattern of RS obtained from incubation with pancreatic α -amylase exhibited a somewhat sharper reflection at 0.537 nm, suggesting slightly higher crystallinity (X-ray diffractograms not shown). In analyzing and interpreting the DSC and X-ray results, it should be noted that DSC and X-ray diffraction, as probes of structural order, do not necessarily measure the same type of structure in retrograded starch (Miles et al 1985b, Russell 1987). The X-ray technique detects regularly repeating ordering of helices, thereby reflecting the three-dimensional order of starch crystallinity. The technique is less sensitive to irregularly packed structures, small chain aggregates, or isolated single helices (Gidley and Bociek 1985). The DSC enthalpy changes are generally considered to correspond to order-disorder transitions of crystallites (i.e., helices present in extended ordered arrays) and regions of lesser crystalline order.

Biliaderis (1991) recently suggested that the DSC thermal responses are directly related to the number of helices present, regardless of their tertiary organization. According to this concept, helices with no crystallographic register also require energy for melting and therefore are thermally detectable. For example, amorphous amylose-lipid complexes (Biliaderis and Galloway 1989) and amorphous amylose-alcohol complexes (Whittam et al 1989), as assessed by X-ray diffraction, have already been shown to give endothermic melting transitions in the DSC thermogram.

In light of these considerations, the higher melting enthalpy but slightly lower crystallinity of RS from Termamyl-amyloglu-

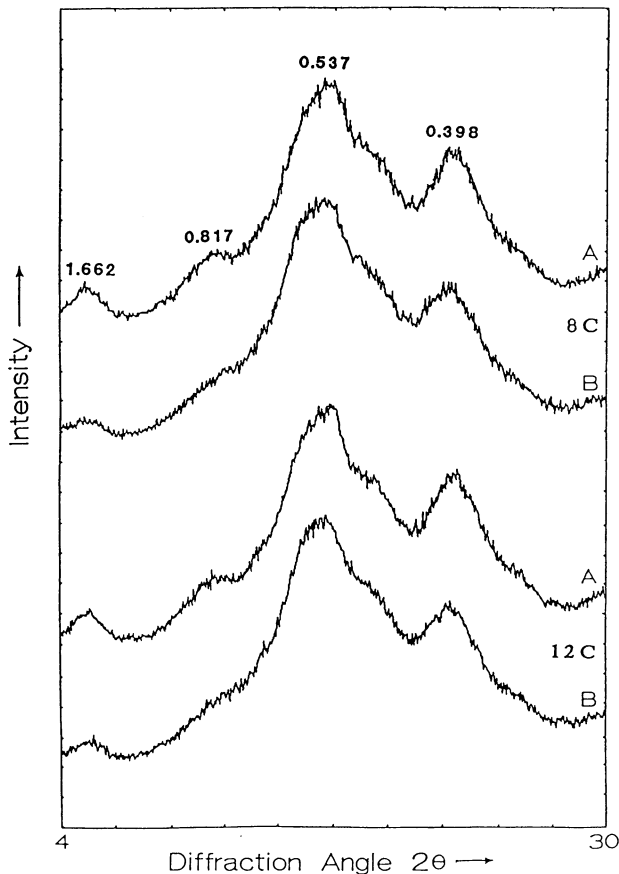


Fig. 4. Effects of repeated autoclaving-cooling cycles (C) on X-ray diffraction patterns of hydrated enzyme-resistant starch (RS). A, ~13% moisture. B, vacuum-dried RS, 4-6% moisture from amylo maize VII starch after 8 and 12C. Numbers above peaks indicate interplanar d-spacings in nanometers.

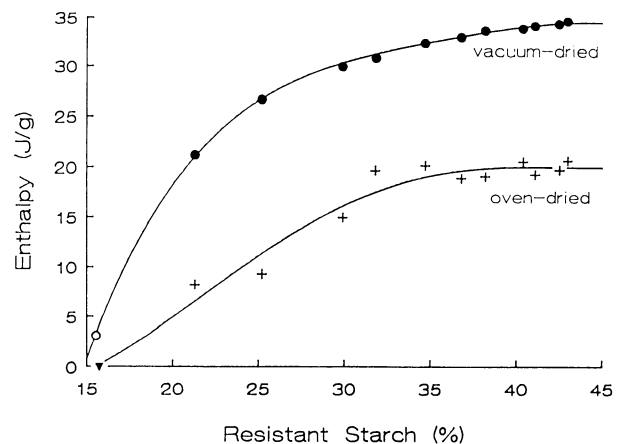


Fig. 5. Effect of vacuum-drying for 48 hr at room temperature and oven-drying for 16 hr at 105°C on melting enthalpies (155°C transition) of enzyme-resistant starch from native amylo maize VII starch (15.8% resistant starch) and autoclaved amylo maize starch samples (21.3-43.0% resistant starch). Open circle and triangle denote enzyme-resistant starch from native amylo maize VII starch.

cosidase treatment, compared with RS from pancreatic α -amylase treatment, could reflect the presence of a higher proportion of retrograded amylose helices that, however, were arranged in less crystalline arrays or in amorphous aggregates.

CONCLUSIONS

X-ray patterns of autoclaved amylo maize starch samples indicated that a structural transformation of the native starch took place during repeated autoclave treatment. The appearance of poor B-type patterns provided evidence that the crystallinity of the altered structure in the autoclaved and cooled preparations was less than the crystallinity of the native starch. Broad peaks at 0.398 nm could be proposed for the unresolved doublet region (0.40–0.37 nm). This is consistent with the general view of a retrograded B-structure showing a broad and diffuse reflection in this region. In view of the decrease in V-structure and increase in B-structure following repeated autoclaving and cooling, amylose from V-type crystallites might play a role in the development of B-type crystallites. Upon cooling of melted amylose-lipid complexes, solubilized amylose chains recrystallized into a V-type structure or precipitated out and became associated with B-type crystallites that were already formed or could be retained as amorphous material. If amylose from amylose-lipid complexes was being incorporated into the B-type crystallites, it could be speculated that such a mechanism contributed to the development of the B-type structure.

Amylolytic treatment of amylo maize starch samples resulted in hydrolysis of amorphous and less resistant crystalline domains and thus concentration of RS fragments. The X-ray pattern of RS residues suggested that chain fragments packed in B-type crystallites with a slightly enlarged crystal lattice contribute to the formation of RS from autoclaved amylo maize starch. RS residues appear to comprise small and/or less perfect crystallites and interspersed amorphous material. X-ray and DSC measurements indicated that RS residues consist of structures that vary in crystallinity and thermal dissociation. Structure development in RS residues after repeated cycles of autoclaving and cooling may be due to increasing interchain amylose associations and to decreasing proportions of less ordered domains.

The procedure used to isolate RS also needs to be considered in evaluating thermal and crystallographic properties of RS. RS isolated after one autoclaving-cooling cycle by pancreatic α -amylase treatment yielded lower melting enthalpies for the 155°C endotherm but slightly higher crystallinity than the corresponding RS fraction isolated by Termamyl-amyloglucosidase treatment. These apparently contrasting DSC and X-ray results were ascribed to differences in the relative proportions of ordered and disordered retrograded amylose chain fragments and to variations in packing of chain fragments in the structures of the two RS residues.

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