Enzyme-Resistant Starch. I. Quantitative and Qualitative Influence of Incubation Time and Temperature of Autoclaved Starch on Resistant Starch Formation

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ABSTRACT

Wheat starch was autoclaved (121°C) for 1 hr in excess water. The subsequent formation of enzyme-resistant starch (RS) was studied as a function of time (at different incubation temperatures, i.e., 0, 68, and 100°C). The rate of formation and the maximum yields of RS (4, 6, and 10%, respectively) varied to a great extent. The relationship between incubation time, temperature, and the yields of RS was interpreted in terms of crystallization in an amorphous matrix, the starch gel being a partially crystalline polymer system. Upon incubation at 0°C, the nucleation rate was high and the propagation rate was low; the opposite was the case upon incubation at 100°C. At 68°C, an intermediate pattern was observed. Incubation of the autoclaved starch samples at two subsequent temperatures (0 and 68°C or 0 and 100°C) (two-step procedure) did not increase the RS yield significantly. X-ray diffraction showed qualitative differences among the crystallites formed at different temperatures. RS formed at 100°C (A pattern) was different from that formed by incubation at 0 or 68°C (B pattern). X-ray diffraction of RS formed in a process with two storage steps at different temperatures (0 and 68°C or 0 and 100°C), aiming at increasing yields by first favoring nucleation and then propagation, yielded B-type diffractograms.

Enzyme-resistant starch (RS) is a physiologically important, indigestible starch fraction. It usually is present in relatively small amounts in food products. Although it is not digested in the small intestine, it may be fermented by microorganisms in the large intestine.

The European Flair Concerted Action on Resistant Starch coordinates research on three types of RS (classified as suggested by Englyst and co-workers [1992]). Type I is physically inaccessible starch, which, when present in a food, is "locked" in the plant cell. Type II is native granular starch found in uncooked starch. Type III is retrograded starch. Other types of RS may also be present in food. Chemically modified starch, such as starch with acetyl or hydroxypropyl substituents, and thermally modified starch can have reduced availability to amylolytic enzymes.

The aim of the present work was to investigate how RS formation is influenced by temperature and storage time (after gelatinization) in an excess of water. During the storage of starch after gelatinization, a fraction becomes resistant for amylolytic enzymes (RS type III) unless it is dispersed, e.g., with $2M$ potassium hydroxide or dimethylsulfoxide. X-ray diffraction of RS type III showed a B-type crystalline structure (Berry et al 1988, Siljeström et al 1989, Sievert et al 1991), and differential scanning calorimetry of RS in water revealed an endotherm transition at about 150°C (Sievert and Pomeranz 1989, 1990; Czuchajowska et al 1991). Results from these and a number of other studies led to the conclusion that RS type III consists mainly of retrograded amylose (Berry 1986; Berry et al 1988; Russell et al 1989; Siljeström 1989; Sievert and Pomeranz 1989, 1990; Czuchajowska et al 1991).

Retrogradation of amylose can be considered as crystallization in an amorphous matrix (starch gel is a partially crystalline polymer system). In general, crystallization consists of three steps (Wunderlich 1976): 1) nucleation, i.e., formation of critical nuclei; 2) propagation, i.e., growth of crystals from the nuclei formed; and 3) maturation, i.e., crystal perfection or continuing slow growth.

The extent to which these processes occur is clearly dependent upon the temperature. Figure 1 shows that the nucleation rate is 0 at the melting temperature of the crystals (T_m); it increases with increasing extent of undercooling ($T_m - T$) or decreasing temperature. At temperatures below the glass transition temperature (T_g), the nucleation rate is negligible; the system is "frozen". The propagation rate is 0 at $T < T_g$ because diffusion does not occur at such temperatures. At higher temperatures, diffusion increases and so does the rate of propagation. At temperatures above T_m, the propagation rate is (for evident reasons) also 0. The maturation rate is dependent on temperature in a way similar to that of the propagation rate (Wunderlich 1976).

The overall crystallization rate depends mainly on the nucleation and propagation rates (Fig. 1). For a partially crystalline polymer system, crystallization can occur only at a temperature between T_g and T_m, i.e., in the rubbery state (Levine and Slade 1988, Marsh and Blanshard 1988). B-type starch gels containing more than 27% water (by weight) have a T_g of about −5°C (Slade 1984), and the T_m of the amylose crystals is about 150°C (Ring et al 1987); therefore, crystallization of amylose can occur only between these temperature limits.

MATERIALS AND METHODS

Wheat starch (Meriwit AA) was supplied by Amylum Aalst (Belgium). Enzymes used for isolation of RS were Termamyl,

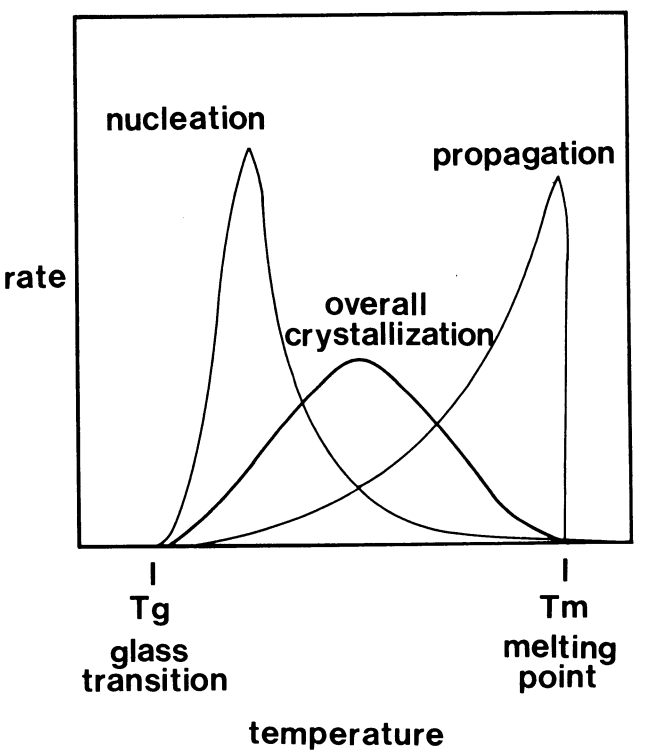

Fig. 1. Dependence on temperature of the nucleation, propagation, and overall crystallization rates of partially crystalline polymers. (Reprinted, with permission, from Levine and Slade 1990)

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a thermostable α-amylase from *Bacillus licheniformis* (Novo Nordisk, Brussels, Belgium); AMG, an amyloglucosidase from *Aspergillus niger* (Novo Nordisk); and protease P5147, from *Streptomyces griseus* (Sigma Chemical Co., St. Louis, MO).

Formation of RS

Starch (1 g) was suspended in 10 ml of water and autoclaved for 1 hr at 121°C. When the temperature had dropped to 100°C, the containers with the starch samples were taken out of the autoclave chamber and immediately transferred to baths at 0, 68, or 100°C.

Isolation of RS

RS was isolated by a modification of the enzymatic-gravimetric procedure for the determination of total dietary fiber (AOAC 1985). At different times, samples were withdrawn from the water bath and 50 ml of boiling phosphate buffer (pH 6.0, 55.6 m*M*) and 0.4 ml of Termamyl were added. After 30 min of incubation at 100°C, the sample was cooled to room temperature and the pH was adjusted to 4.5 with a 2% phosphoric acid solution. Amyloglucosidase (1 ml) was added and the sample was incubated for 30 min at 60°C. After enzymic digestion, the sample was centrifuged (10 min, 1,000 × *g*) and the sediment was washed three times with distilled water. The residue was resuspended in 50 ml of phosphate buffer (pH 7.5, 90.9 m*M*). Protease was added (1 ml of a solution containing 16 mg of protease in 100 ml of buffer) and the residue was incubated for 4 hr at 42°C. The sample was filtered through a weighed fritted crucible (no. 4 porosity) and washed three times with distilled water. The crucible with the residue was dried overnight in an air oven at 80°C and weighed after cooling to room temperature in a desiccator. Thus, RS was determined as the insoluble residue after enzymatic digestion of the starch sample after removal of the amylolytic enzymes with protease. Results are expressed as percent RS on a dry matter basis.

X-Ray Diffraction

X-ray power diffraction analysis was performed with a PW 10050/25 diffractometer (Philips, MBLE, Brussels, Belgium) operating at 40 kV, 20 mA, with CuK radiation = 0.154 nm (nickel filter). Diffractograms of dried RS samples were 2° 2θ to 30° 2θ.

RESULTS AND DISCUSSION

In this study, RS refers to the insoluble residue obtained under in vitro conditions as described above. The RS obtained under in vitro conditions in our work should not necessarily be the same as RS obtained under in vivo conditions. This is not only because of the different pH and temperature conditions of the assay (100°C is far removed from physiological conditions), but also because the enzymes are not the same. Apart from the obvious (practical) advantages associated with the use of our procedure, our approach has the definite advantage of easily comparing our results with those by other authors (Björck et al 1987; Siljeström et al 1989; Sievert and Pomeranz 1989, 1990; Sievert et al 1991; Czuchajowska et al 1991).

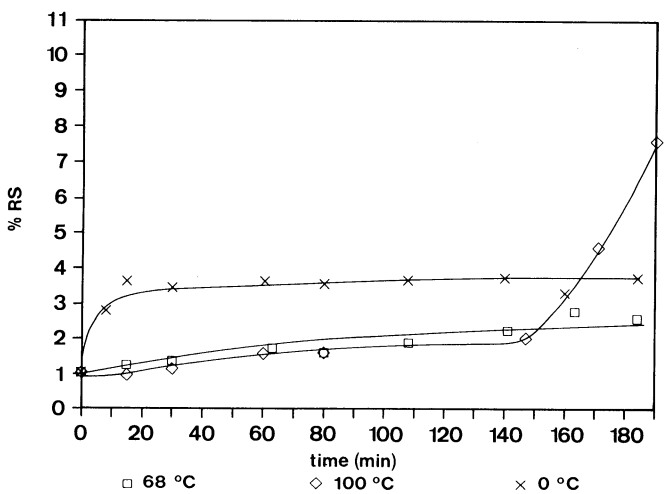

Fig. 2. Enzyme-resistant starch content (%RS) in starch-water mixtures autoclaved for 1 hr at 121°C and stored at different temperatures (0, 68, and 100°C) as a function of time (first 200 min).

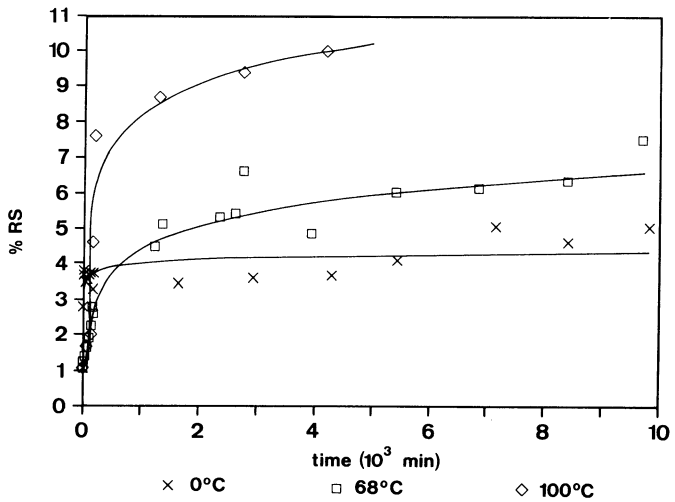

Fig. 3. Enzyme-resistant starch content (%RS) in starch-water mixtures autoclaved for 1 hr at 121°C and stored at different temperatures (0, 68, and 100°C) as a function of time (10 × 10³ min).

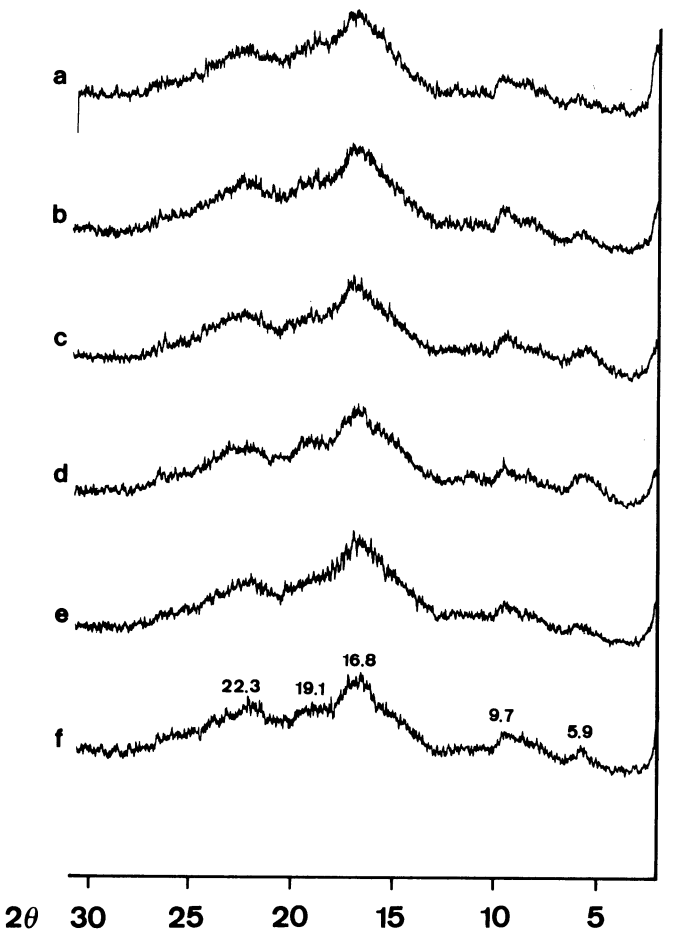

Fig. 4. X-ray diffraction patterns of isolated resistant starch formed in an autoclaved starch-water mixture at 0°C after 80 min (**a**), 160 min (**b**), 1,600 min (**c**), 2,900 min (**d**), 5,400 min (**e**), and 9,800 min (**f**). Numbers above peaks indicate reflection angles.

Formation of RS in Autoclaved Starch-Water Mixtures Stored at 0, 68, and 100°C

Data are shown as yields of RS (%RS on a dry matter basis) as a function of incubation time. The data are fitted to a logarithmic function as: $\%RS = a_0 + a_1 \ln(\text{time})$. This is a convenient way to represent the data without implication regarding the mechanism of RS formation.

The changes in RS content of the starch-water mixtures autoclaved for 1 hr at 121°C and stored at different temperatures (0, 68, and 100°C) with storage time are presented in Figure 2 (first 200 min) and Figure 3 (up to 10×10^3 min). At all storage temperatures, we observed two stages in the RS formation. The amount of RS formed initially increased quickly (although a lag phase was observed at 100°C) but leveled off with time. Both the initial rate of RS formation and the maximum yield that could be obtained varied, to a great extent, with the incubtion temperature. At 0°C, the initial rate was very high; after 15 min, the maximum yield (about 4%) of RS under such conditions was formed. At 68°C, the initial formation of RS was slower, but the maximum yield formed at this temperature was higher (about 6%). At 100°C, there was an induction time of about 150 min before the RS yields quickly increased to a maximum (about 10%), which was higher than the maximums obtained at storage temperatures of 0 and 68°C.

RS Formation in Starch Gel: A Crystallization Process in a Partially Crystalline Polymer System

The relationship between storage time, temperature, and amount of RS formed could be interpreted in terms of crystallization in a partially crystalline polymer system. It is logical that the amount of RS formed depended on the overall crystallization, which, in turn, is a function of nucleation and propagation. Indeed, both nucleation and crystal growth were affected by temperature.

At 0°C, the extent of undercooling ($T_m - T$) for the formation of amylose crystals is very high (150–0°C). Thus, the nucleation rate is very high. The initial yield of RS increases rapidly. On the other hand, the difference between T_g (−5°C) and 0°C is very small. The growth of the formed crystal nuclei was limited because of the high viscosity. Many nuclei were formed very quickly, but they only had a limited, if any, potential to grow further into crystals (Figs. 2 and 3).

At 100°C, the nucleation was limited but the propagation was favored. Under these conditions, it took some time before nuclei were formed. However, once a limited number of nuclei were formed at this temperature, they grew very quickly. The induction zone of the curve at 100°C was, therefore, accompanied by a rapid increase of the RS yield. The maximum yield of RS formed was even higher than that at the lower storage temperatures. This RS fraction probably consisted of a more limited number of large crystals.

At the storage temperature of 68°C, we found an intermediate pattern. The nucleation rate was slower than that at 0°C and faster than that at 100°C. The propagation rate was faster than that at 0°C and slower than that at 100°C. The overall crystallization, or the amount of RS formed, depended on both the nucleation and the propagation rates.

X-Ray Diffraction of Isolated RS Formed at 0, 68, and 100°C

The X-ray diffractograms of the isolated RS formed by storage at 0, 68, and 100°C are shown in Figures 4–6, respectively. The diffractograms of RS obtained by storage of the gelatinized starch at 0°C for increasing storage time were essentially the same. For the RS formed at 68°C, we noted a slight increase in crystallinity with increasing storage time. For the RS formed at 100°C, the increase in crystallinity was very obvious. As stated above, fewer but larger crystals were formed at these higher temperatures. Furthermore, if a maturation process had taken place, it would

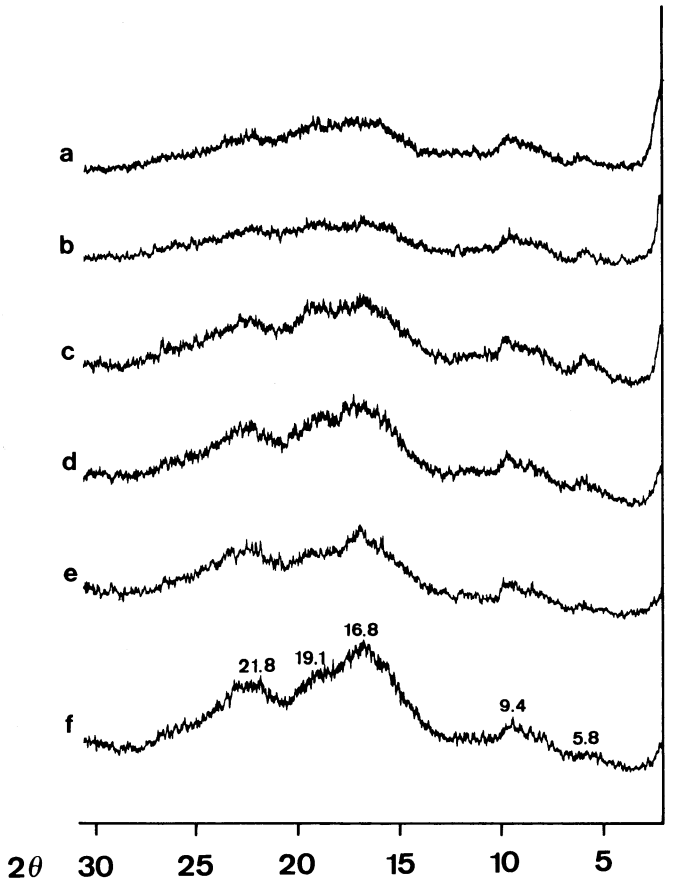

Fig. 5. X-ray diffraction patterns of isolated resistant starch formed in an autoclaved starch-water mixture at 68°C after 60 min (**a**), 160 min (**b**), 1,200 min (**c**), 2,400 min (**d**), 5,400 min (**e**), and 9,700 min (**f**). Numbers above peaks indicate reflection angles.

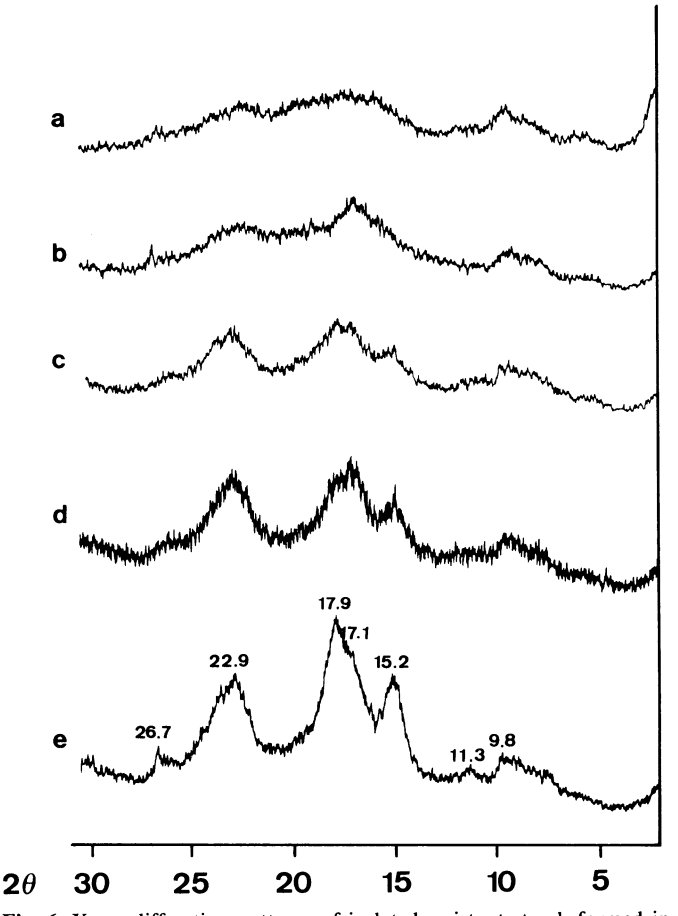

Fig. 6. X-ray diffraction patterns of isolated resistant starch formed in an autoclaved starch-water mixture at 100°C after 60 min (**a**), 170 min (**b**), 190 min (**c**), 1,300 min (**d**), and 2,800 min (**e**). Numbers above peaks indicate reflection angles.

have been more pronounced at the higher temperatures because the maturation rate increases with increasing temperature.

The level of crystallinity of the isolated RS fractions increased with increasing storage time. It is not unlikely that isolated RS fractions still contain amorphous parts locked within the crystalline regions that are inaccessible for amylolytic enzymic action. One can imagine that, with increasing storage time of the starch gel, these amophous parts would gain crystallinity.

Furthermore, the X-ray pattern of the RS formed at 100°C was very different from the pattern formed at lower temperatures. At 0 and 68°C, the X-ray diffractograms showed a B-type crystalline structure (Table I, Figs. 4 and 5). These observations agree with data by other authors. Berry et al (1988) prepared RS from wheat starch by autoclaving at 134°C (45 min), drying, and incubating with α-amylase and pullulanase. Siljeström et al (1989) isolated RS from wheat starch with Termamyl, pepsin, pancreatin, and amyloglucosidase after autoclaving (1 hr), cooling to room temperature, and lyophilizing. Sievert et al (1991) prepared RS from amylomaize VII starch by autoclaving at 121°C (1 hr), cooling at 4°C (overnight), and incubating with Termamyl and amyloglucosidase. X-ray diffraction analysis of these RS samples formed under different conditions all showed a B-type X-ray diffraction pattern.

The X-ray diffraction pattern of RS formed at 100°C resembled the A-type starch (Table II, Fig. 6). Earlier authors have demonstrated (with debranched glycogen) that a higher crystallization temperature generally favors the formation of the more stable A-type, rather than B-type, starch polymorph (Gidley 1987, Gidley and Bulpin 1987). Lower temperatures are expected to favor the polymorphic form requiring the least entropy change (thus, the least activation energy) from solution (B-type), i.e., the kinetic product. At higher temperatures, crystallization tends to favor the more stable polymorph (A-type) requiring a higher activation energy, i.e., the thermodynamic product (Gidley 1987).

TABLE I
B-Type Crystal Polymorphs and Interplanar Spacings Deduced from X-Ray Power Diffraction Patterns of Isolated Resistant Starch (RS) Formed at Different Storage Temperatures

Interplanar Spacings (Å)[a,b]	Deduced Interplanar Spacings, Å	
	0°C	68°C
15.8 m	(15.78–14.49) m	(15.50–14.98) m
8.90 w–	(9.61–9.03) m	(9.61–9.03) m
7.94 w–	(8.04–7.56) w–	(8.12–7.50) w–
6.14 m	...	...
5.16 s	(5.31–5.19) s	(5.34–5.16) s
4.54 w+	(4.80–4.58) m	(4.75–4.55) m
4.00 m	(4.04–3.93) m	(4.08–3.90) m
3.70 m–	...	...
3.38 w	(3.41–3.33) w	(3.38–3.35) w
2.60 w	...	...

[a]Zobel 1964 (with permission).
[b]Intensity scale s = strong, m = medium, w = weak, – = less than, + = more than.

TABLE II
A-Type Crystal Polymorphs and Interplanar Spacings Deduced from X-Ray Power Diffraction Patterns of Isolated Resistant Starch (RS) Formed at Different Storage Temperatures

Interplanar Spacings (Å)[a,b]	Deduced Interplanar Spacings, Å
	100°C
...	(16.37–14.73) w
8.72 w–	(9.61–8.93) m
7.70 w–	(7.97–7.63) w–
5.78 s	(5.87–5.68) s–
5.17 s	(5.22–5.10) s
4.86 s–	(4.98–4.96) s
4.37 m	(4.67–4.48) w–
3.78 s	(4.00–3.85) s
3.30 w+	(3.45–3.33) w
2.88 w	...

[a]Zobel 1964 (with permission).
[b]Intensity scale s = strong, m = medium, w = weak, – = less than, + = more than.

Formation of RS in Autoclaved Starch-Water Mixtures Stored at Two Subsequent Temperatures (0 and 68°C, or 0 and 100°C)

We tried to obtain a maximum yield of RS in a minimum of time by nucleation at 0°C followed by propagation at a higher temperature (68 or 100°C). We expected the greatest yields for incubation at 0°C (30 min) and subsequent storage at 100°C. From our previous results, it was clear that at 0°C the RS content increased rapidly in the early stages of the experiment (nucleation favored); at 100°C the yield of RS became significant after an induction time (propagation favored) (Figs. 2 and 3).

However, we noted that the yield of RS formed at 100°C after incubation at 0°C for 30 min did not increase much (Fig. 7). Even after incubation at 100°C for times much longer than the induction time (about 150 min) for RS formation at 100°C without prior incubation at 0°C, the amount of RS did not increase. Incubation of autoclaved water-starch mixtures at 68°C after storage at 0°C did not increase the RS yield significantly either (Fig. 7).

These observations can be rationalized if one assumes that at 0°C the autoclaved starch-water mixture forms a strong gel very quickly. When this gel is warmed to 68°C or even 100°C, its strength is still higher than that of an autoclaved starch-water mixture that has been continously kept at 68 or 100°C. Miles et al (1985) showed that the shear modulus of starch gels stored for several days, heated to 95°C, and quickly cooled to 25°C drops to the value that it had after 24-hr storage at 25°C, whereas that of an amylose gel remained unchanged. The increase of the shear modulus for long-term (over 24 hr) stored gels can be reversed by heating to 95°C; this is in contrast with the increase of the shear modulus for short-term (less than 24 hr) stored gels that is contributed by the amylose fraction. In our experiments, the starch gels had been incubated only for 30 min at 0°C and for a maximum of 210 min at 68 or 100°C. It is reasonable to assume that amylose is responsible for the gel strength of the autoclaved starch-water mixture and that this strength cannot be reversed by heating to 95°C after incubation at 0°C.

It is further reasonable to expect that diffusion of amylose molecules to nuclei or crystals in a high-strength gel is very difficult. A further increase of RS yields after incubation of an autoclaved starch-water mixture at 0°C can be expected to be very slow.

Comparison of the RS yields after subsequent incubation at 0 and 100°C and at 0 and 68°C showed a slightly lower yield of RS for the samples incubated at 0 and 100°C for about the first 100 min. After this period, the yields are the same for the different temperatures. This phenomenon may be related to the

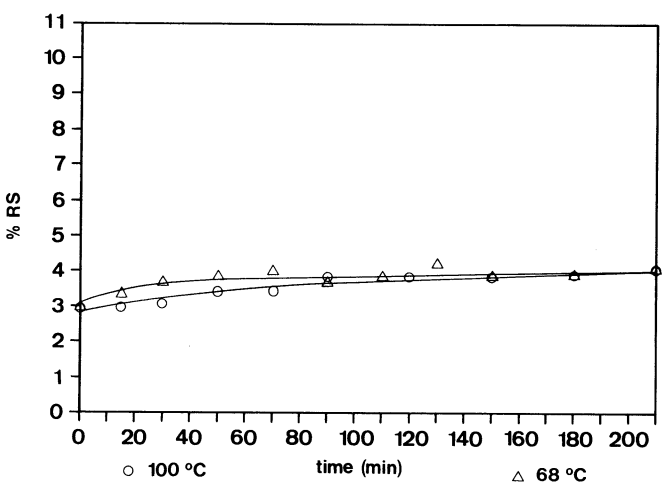

Fig. 7. Enzyme-resistant starch (%RS) content in autoclaved starch-water mixtures stored at 0°C for 30 min and at 68 or 100°C for a varying time.

different crystal types of the RS formed at 0°C and at 100°C (B-type and A-type, respectively, Figs. 4 and 6). In contrast, the same crystal type (B-type) of the RS is formed at 0°C and at 68°C. It seems likely that growth of the nuclei or crystals formed at 0°C, which show a B-type X-ray diffraction pattern, is more difficult at 100°C, where an A-type pattern is favored, than at 68°C, where a B-type is favored. Also, the formation of new seeds of the A-type crystals at 100°C would probably take even longer during incubation at 100°C. This is because the concentration of amorphous polymers available for crystallization has decreased after incubation at 0°C.

These results demonstrate that a two-step procedure with subsequent incubation at 0°C and a higher temperature is not the best way to achieve a high amount of RS in a relatively short time. A higher amount of RS (about 10%) for wheat starch can be obtained by a single-step procedure at 100°C, but it requires storage times of three days or more. These findings are in contrast with results for amylopectin crystallization. Slade (1984) has shown the greatest amounts of amylopectin recrystallization by a two-step procedure at 4°C (nucleation) and 40°C (propagation). These temperatures are slightly above the T_g of the starch gel (−5°C) and below T_m of amylopectine crystals (~60°C), respectively.

The different observations may be due to the different crystallization processes of amylopectin and amylose. Crystallization of amylopectin is believed to take place in the swollen gelatinized granules, whereas amylose crystallizes in the amylose gel matrix. The viscosity of the starch gel probably has more influence on the diffusion of amylose chains (25% for wheat starch) in the matrix than it does on the diffusion of the amylopectin chains (75% for wheat starch) in the granules. Apparently, the formation of a strong starch gel at 0°C does not affect the amylopectin crystallization as much as it affects the amylose crystallization.

X-Ray Diffraction of Isolated RS Formed at 0°C Followed by a Higher Temperature (68 or 100°C)

The X-ray diffractograms of isolated RS formed at temperature combinations of 0°C followed by 68 or 100°C are shown in Figures 8 and 9. The patterns were essentially the same: they all showed a B-type pattern. Because the X-ray pattern of the RS formed at 0°C followed by 100°C had a B-type pattern, we know that the B-type nuclei or crystals formed at 0°C had not changed into A-type. Also, there were no new A-type nuclei or crystals formed at 100°C that could be detected in the diffractograms.

CONCLUSIONS

Formation of RS in a starch gel can be considered crystallization of amylose in a partially crystalline polymer system. Thus, RS yields largely depend on storage time and on storage temperature (between T_g and T_m). Nucleation is favored at temperatures far below T_m of the crystals but above T_g, but propagation is limited under these conditions. At temperatures far above T_g but below T_m, propagation is favored and nucleation is limited.

The storage temperature influences the type of RS crystal (A or B X-ray diffraction pattern) formed. The crystallinity of the isolated RS increases with storage time at higher storage temperatures (68 and 100°C).

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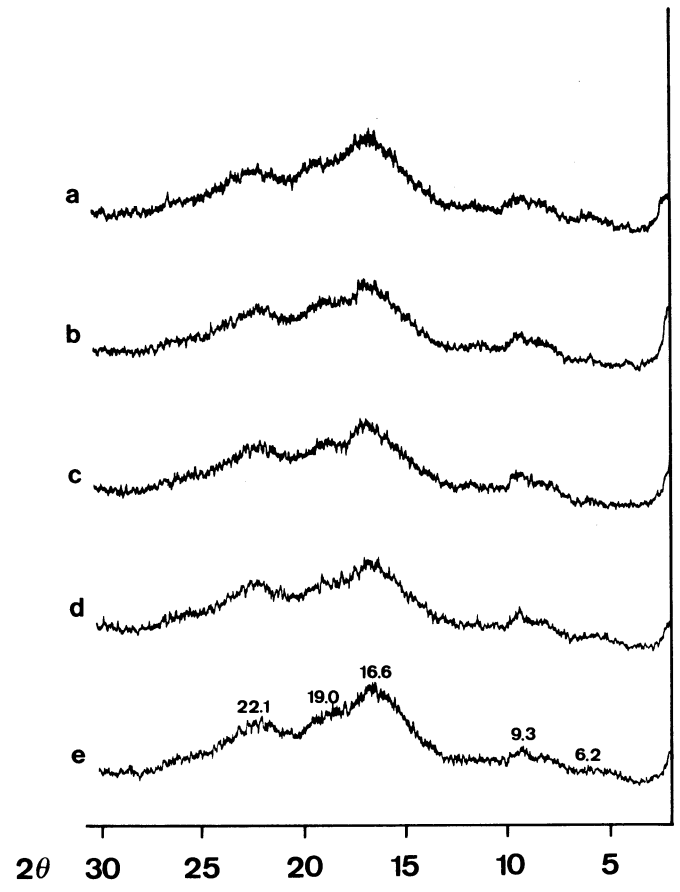

Fig. 8. X-ray diffraction patterns of isolated resistant starch formed in an autoclaved starch-water mixture at 0°C for 30 min and at 68°C for 30 min (**a**), 70 min (**b**), 100 min (**c**), 170 min (**d**), and 210 min (**e**). Numbers above peaks indicate reflection angles.

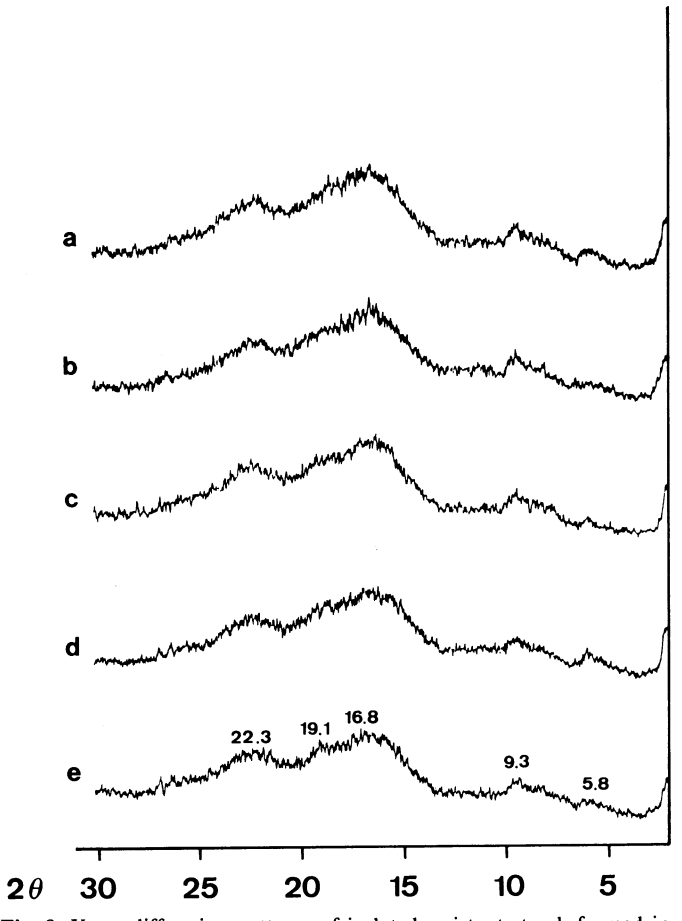

Fig. 9. X-ray diffraction patterns of isolated resistant starch formed in an autoclaved starch-water mixture at 0°C for 30 min and at 100°C for 30 min (**a**), 70 min (**b**), 100 min (**c**), 170 min (**d**), and 210 min (**e**). Numbers above peaks indicate reflection angles.

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