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# Modification of amylose and investigation of its inclusion behavior

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**Abstract**

Five different amylose derivatives—hydroxypropyl, hydroxyethyl, 1,2-dihydroxypropyl, acetyl and carboxymethyl amylose—with varying degrees of substitution were investigated concerning their water solubility and their complexing ability of iodine and of several organic compounds. In general, the water solubility of all samples increased with increasing degree of substitution, whereas their complexing ability decreased. This was examined by means of UV spectroscopy and circular dichroism. The three derivatives with substituents carrying hydroxy groups showed almost equivalent behavior, while acetylated amyloses revealed much more guest-specific complexing properties. A reduced complexing ability of the carboxymethylated amyloses is attributed to an enhanced substitution at the HO-3 and HO-6 groups disturbing the helical conformation of the amylose chain and lowering the complex stability. In addition, the pH dependence was determined for two samples of carboxymethyl amylose. 

*Keywords:* Amylose derivatives, water solubility of; Organic compounds, inclusion of; Spectra of iodine complexes; Circular dichroism of amylose complexes

## 1. Introduction

The use of native amyloses and starches in food industry and technical chemistry for certain applications is limited by their specific physical properties. Whereas their ability to form gels and films is of great value, the low water solubility and the tendency to retrograde at low temperatures or longer storage is a disadvantage for some applications. As it will be shown in this paper, these properties can be improved by derivatisation and modification of starches and adapted for the desired requirements.

Starches from different sources contain amylopectin and amylose in different proportions. Inclusion of organic compounds in the helical segments of amylose normally yields insoluble complexes. This fact is often utilized to fractionate starch samples and to separate amylose from amylopectin [1]. A complete separation of amylose from amylopectin is not possible. We have used amylose isolated by repeated, selective precipitation with cyclohexane from a potato starch. Potato amylose is known to be a highly linear $\alpha$-(1→4)-linked glucose polymer containing only a few long

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chain branches. The extent of branching has not been investigated in detail.

To investigate the physical properties and the complexing ability of amylose in soln, we wished to use various spectroscopical methods and therefore required soluble complexes. Soluble complexes of amylose would also be of special interest in certain technical applications. Thus otherwise sparingly water-soluble bioactive compounds might be obtained in water-soluble form by complexation and be used for different applications. In order to obtain soluble amyloses and amylose inclusion complexes, we investigated systematically the influence of the type and degree of substitution in the amylose molecules on these properties.

Modification with hydroxypropyl groups is especially interesting. Due to their properties and toxicological safety, hydroxypropylated starches have found many applications in food, e.g. as thickeners, stabilizers and water-binding compounds [2]. They are also used in paper production and textile industry. Here their advantageous film-forming properties, especially the clearness and flexibility of films consisting of hydroxypropylated starches, is of great importance [3].

In previous investigations [4,5] we were able to show that an average molar degree of substitution (m.d.s.) of 0.06–0.075 of hydroxypropyl groups in amylose is sufficient to obtain soluble inclusion complexes with weak organic complexing agents. However, guest molecules which were able to establish very strong molecular complexes need higher substituted amyloses as hosts to ensure satisfactory water solubility. In these cases a m.d.s. $\geq$0.13 can be necessary. The degree of substitution (d.s.) is defined as the average number of substituted hydroxy groups per anhydroglucose unit, the molar degree of substitution (m.d.s.) is defined as the average number of moles of alkylene oxide combined per anhydroglucose unit. This difference exists in modifications with oxiranes since substitution on already substituted positions, e.g. on the OH group of a hydroxypropyl-substituent, is possible. With low degree of substitution (0.04–0.7) d.s. and m.d.s. are nearly identical. Only at higher degree of substitution the differences become significant. Hence, the highest obtainable d.s. of starch products is three, the m.d.s. can reach much higher values.

Hydroxypropylation is usually carried out in alkaline solution using propylene oxide as reagent. To prevent oxidative degradation, it is advisable to work under inert conditions. In principle, all three hydroxyl groups at C-2, C-3 and C-6 of the anhydroglucose units can react, but mainly substitution at HO-2 is observed [6,7]. For a hydroxypropylated amylose with a m.d.s. of 0.077 substitution of 5.23 mol% at HO-2, 0.51 mol% at HO-3, and 2.03 mol% at HO-6 was determined [8]. The reason for this preference is an enhanced reactivity due to the proximity of the C-2 position to the anomeric center. Substitution of HO-6 occurs in significant amounts only at higher pH or higher degrees of substitution; the C-3 position is generally less reactive. As Hui et al. [9] reported, substitution of HO-2 does not destabilize the helical conformation considerably.

## 2. Results and discussion

*Modification of amylose*.—The intention of the present investigations was to determine the properties of various derivatized amyloses. Especially their solubility and complexing abilities should be compared.

It was shown before [4,5] that hydroxypropylation to a low m.d.s. increased the solubility of amyloses significantly without affecting their complexing ability too much. Nevertheless, it is of great interest to examine whether or not other substituents may be even more suitable to satisfy these high demands.

On account of the good water solubility of hydroxypropyl starches, it should be examined how hydroxyethyl derivatives behave and whether or not a second hydroxyl group bound to the substituent would increase the solubility even more. Moreover, we investigated in which way the complexing behavior of such modified amyloses would be affected. We therefore used propylene oxide, ethylene oxide and 2,3-epoxy-1-propanol (glycidol) for amylose modifications (Fig. 1).

Several samples of all three amylose derivatives with various m.d.s. were synthesized using commercially available potato amylose from Avebe (The Netherlands). The starting amylose shows a $M_w = 420{,}000$ and a $M_n = 240{,}000$ which results in a relatively narrow molecular weight distribution of $M_w/M_n = 1.75$. These values were obtained by g.p.c. using a light scattering detector. They were investigated for their water solubility and their complexing abilities to iodine and to different organic molecules.

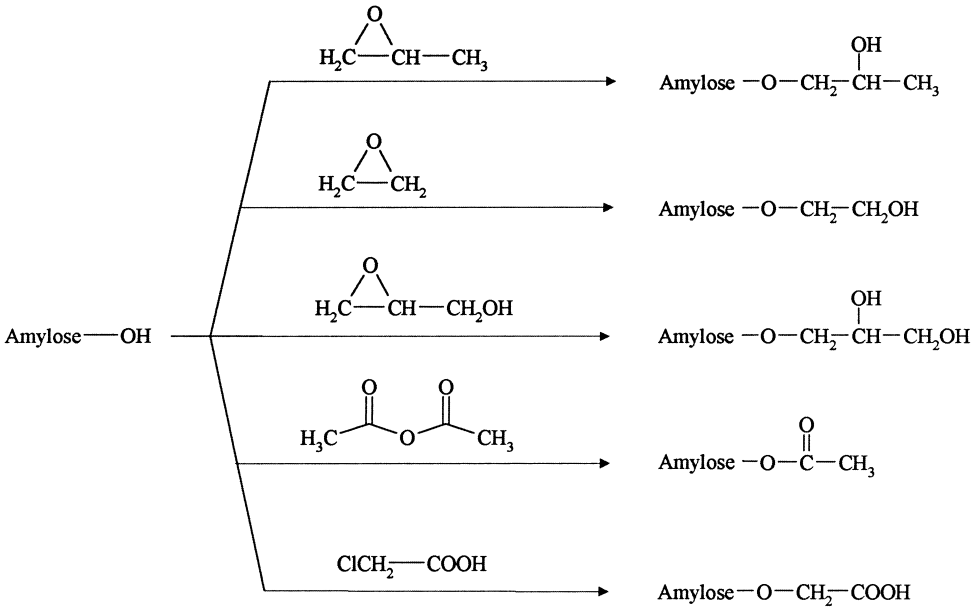



Fig. 1. Preparation of amylose derivatives.

In comparison to the derivatized amyloses where the substituents carry additional hydroxy groups, we synthesized several acetyl amyloses by substitution of amylose with acetic anhydride. The acetyl group has an even lower sterical demand than the hydroxypropyl group and should therefore disturb the helical conformation and the complexing capacity of the amyloses to a lesser extent.

To obtain ionic derivatives of amylose for comparison with the uncharged polymers, we synthesized various samples of carboxymethyl amylose by modification of dissolved amylose with chloroacetic acid [10,11] as an example for anionic carbohydrates. Besides the degree of substitution, the influence of charge and, hence, of the pH was of special interest. A much higher water solubility was expected for the sodium salt of the carboxymethyl-substituted amylose compared to uncharged samples, thus requiring a much lower degree of substitution for soluble complexes to be obtained.

In case of substitution with acetyl or carboxymethyl groups no difference in d.s. and m.d.s. is expected even with higher degree of substitution, since no further reaction at the substituents is possible. Direct comparison between d.s. of acetyl and carboxymethyl with m.d.s. of the other substituent containing hydroxy groups is allowed since with the exception of one case all samples contain m.d.s. values below 0.8.

Altogether eight samples of hydroxypropylated amyloses with different molar degrees of substitution were synthesized. In addition, three samples of 1,2-dihydroxypropyl and two samples of hydroxyethyl amyloses were prepared. In all cases the m.d.s. was determined by evaluating the $^1$H NMR signals belonging to the anomeric protons of the anhydroglucose units (AGU). Since the peaks of those anomeric protons which are situated close to a substituent group are shifted slightly downfield following an electronical deshielding effect, the m.d.s. can easily be determined by comparing the intensities of the corresponding NMR signals. The results are in good agreement with those obtained by chemical analysis of hydroxypropylated amyloses [7].

Fig. 2 shows the relation between the applied quantities of propylene oxide and the spectroscopically determined molar degrees of substitution for the hydroxypropylated amyloses. As can be seen in Fig. 2, a linear dependence exists between these two parameters.

The d.s. of the acetylated amyloses can also be easily determined by means of $^1$H NMR spectroscopy. Here the acetyl methyl groups provide a clear indicator of the amount of substituents bound to the polymer.

To determine the degree of substitution of the carboxymethyl derivatives, we transformed the carboxyl groups of the substituents quantitatively into the acid form by treatment with an ion-exchange resin. Afterwards, the polymers were dried and titrated against 0.1 M NaOH.

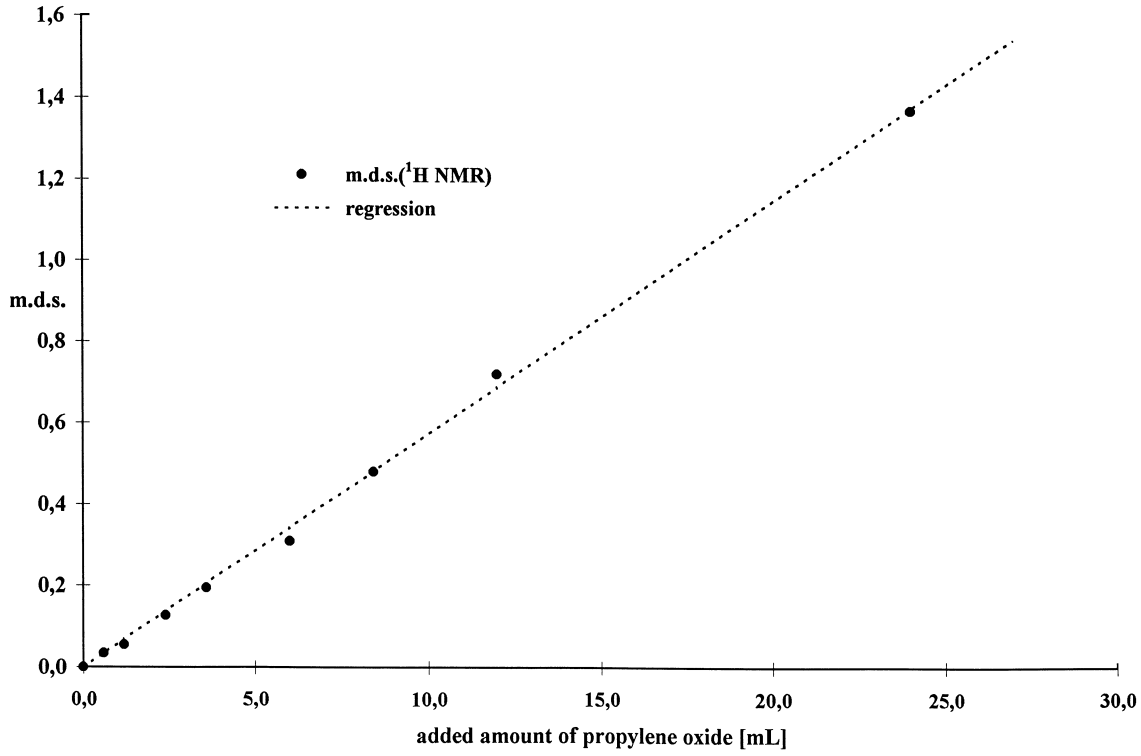



Fig. 2. Dependence of the degree of substitution of the hydroxypropyl amyloses on the added amounts of propylene oxide.

*Characterization of the solubility and complexing ability of the substituted amyloses.*—For the characterization of the complexing behavior of starch products, measuring the absorption of the iodine-complex is an appropriate and wide-spread method. Hence, the complexing ability can be expressed by the blue value (BV). High blue values indicate long polyiodide arrangements and thus extended helical segments within the amylose chains and may be used as evidence for good complexing ability. To determine the blue value, we mixed an amylose solution of known concentration with a specific amount of a 0.2% $I_2$/KI soln and stored it in the dark for a defined period. Afterwards, the extinction of the solution at 680 nm is measured and the blue value is calculated using the following equation, which was established by Gilbert and Spragg [12] as an empirical equation:

$$BV = \frac{0.4 \cdot E}{c}$$

$E$ = extinction at 680 nm; $c$ = concentration of the soln (mg amylose/mL solution). A 0.002% solution of $I_2$/KI is used as reference.

Moreover, the wavelength at maximum absorption of the iodine complexes should be an indicator for the extension of helical segments within the amylose molecules. The longer the polyiodine chains in the helical segments of the amyloses are, the more the absorption maximum shifts to higher wavelengths [13,14]. The iodine complexation is mainly influenced by the degree of substitution and to some extent by the type of substituent. Higher substitution disturbs the formation of helices since hydrogen bonding between HO-6 of a glucose unit of one helix turn to HO-3 of a glucose unit of the next turn is not possible in case of substitution of these OH-groups. According to investigations of Hui et al. [15] this hydrogen bonding is the main stabilizing factor for helix formation. Since the different substituent groups at the anhydroglucose units disturb the helical conformation in a more or less extensive manner, this value is of great interest in comparing the various modifications.

Fig. 3 shows the iodine spectra of native and some hydroxypropylated amyloses of different molar degree of substitution. The left side of the diagram point out the two peaks of free iodine at 288 and 350 nm, whereas the broad absorption at the right side represents the complexed portions. To demonstrate the decreasing amount of free iodine in the solutions with increasing quantity of bound iodine in dependence of m.d.s., we used an lesser concentrated iodine solution as reference, possessing an extinction of approximately 1.

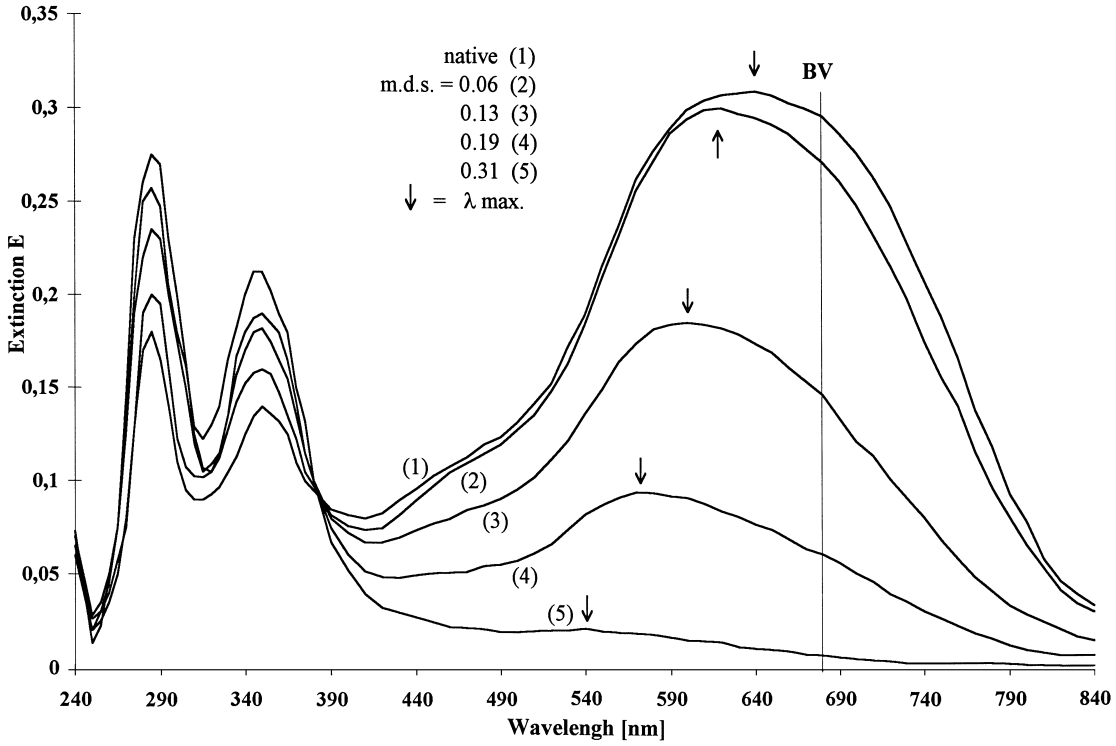



Fig. 3. Iodine spectra of hydroxypropylated amyloses with different degrees of substitution (m.d.s.).

Furthermore, the amount of complexed iodine can be calculated by measuring the decrease of extinction of free iodine at 288 nm in the complex solutions [16]. The complexed quantity is obtained by determining the difference in extinction to the reference solution of free iodine, expressed as mg iodine/g amylose [17].

Accordingly the conventional blue value at 680 nm as well as the wavelength of maximal absorption and the amount of encapsulated iodine was determined.

In complexing organic compounds it was of interest to determine the amount of a substance that can be solubilized by molecular encapsulation in amylose. Fenchone was chosen as organic guest molecule because it forms very stable, insoluble inclusion complexes with unmodified amylose. It was our aim to investigate the degree of substitution that is necessary to obtain soluble complexes and to probe the influence of various substituents on the complexing behavior. Hence, it was examined how much fenchone could be solubilized by the given amylose solutions. For this, amylose solutions of various concentrations were investigated. The highest concentration examined was 5% w/w since higher concentrated solutions became too viscous. Besides, in this way aggregation and intermolecular interactions should be excluded as much as possible.

The quantity of dissolved fenchone[1] was determined from the absorption maximum of the carbonyl group of the monoterpene at 278 nm ($n\rightarrow\pi^*$ transition, $\varepsilon = 27$ L/mol. cm). The water solubility of fenchone was determined by a calibration curve. The obtained value (2.31 g/L) was in good agreement with literature data [19].

CD spectroscopy is another suitable method for investigating the complexing behavior of water soluble amylose derivatives. As described before for cyclodextrin complexes [20,21], achiral guest molecules with a chromophore may show inside a chiral amylose helix an induced Cotton effect, which can be determined CD-spectroscopically at the absorption maximum of the chromophore. The iodine complex is the best known example for amylose complexes. Inclusion of iodine into the amylose helix induces several strong Cotton effects [22,23].

Beyond this, extended investigations have shown that numerous organic complexing agents give induced Cotton effects with hydroxypropylated amylose [5].

[1] Lit. data: $\varepsilon = 21.3$\; L/mol\,cm; $\lambda_{max} = 285$\; nm (96% ethanol) [18].

The extinction of the observed Cotton effects in spectra of soluble complexes, containing different amylose derivatives with the same guest molecule, should be proportional to their complexing ability. 4-Methyl-2-pentanone and 4-*tert*-butylphenol were chosen as guest molecules for complexation. 4-Methyl-2-pentanone is a rather slim molecule which is also preferably complexed by $\alpha$-cyclodextrin. The amylose molecule adapts in this complex the energetically favored helical conformation with six anhydroglucose units (AGU) per turn. 4-*tert*-Butylphenol is a much more sterically extented molecule. Accordingly it is preferably complexed by $\beta$-cyclodextrin. In complexation with amylose derivatives an enlargement of the amylose helix to seven AGU per turn is required [24–26] (Fig. 4).

Consequently, the corresponding CD spectra also reveal the modified amyloses' ability to enlarge the diameter of the helix.

The results of the investigations concerning the solubility measurements and the complexing abilities for the five different amylose derivatives are listed in Tables 1 and 2.

The results in Tables 1 and 2 show a considerable increase in water solubility with increasing degree of substitution for all amylose derivatives (m.d.s. investigated between 0.04 and 1.37). The higher substituted products are all water-soluble without heating the solution. Especially the amyloses that were treated with 2,3-epoxy-1-propanol (glycidol) show a particularly enhanced solubility.

Compared to hydroxypropylated amyloses of the same degree of substitution, the water solubilities of the acetylated amyloses are nearly equivalent. The chemical nature of the substituents with no hydroxyl functions seem to be of no significant importance in this context.

Examining the carboxymethyl derivatives, the influence of the carboxylic acid function on water solubility is surprisingly lower as supposed. The obtained solubilities are only slightly improved compared to the amyloses carrying substituents with hydroxyl groups.

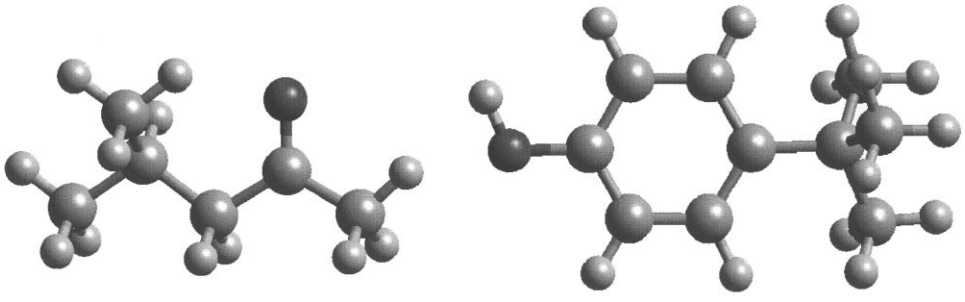
On the other hand, the blue values of all samples decrease distinctly with increasing degree of substitution. In addition to this, the maximum absorption of the iodine complex shifted significantly to shorter wavelength indicating the inclusion of shorter iodine chains into the amylose helix. The determined amount of complexed iodine decreases in the same manner, so the ability of complexing iodine becomes remarkably reduced with increasing d.s.. So these three factors show a similar dependence on the degree of substitution as shown in Fig. 5 for the hydroxypropylated samples.

The decrease of complexing ability is obviously due to the introduction of substituents into the polysaccharide. Probably, the substituents disturb the development of extended helical segments within the amylose chains; at least, they disturb the inclusion of prolonged coherent iodine chains into the helix.

However, samples of the same m.d.s. of hydroxyethyl, hydroxypropyl and 1,2-dihydroxypropyl amylose show equivalent blue values and comparable iodine binding capacities. It is therefore obvious that amyloses modified with these three substituents are likewise capable of complexing iodine.

Unlike to this, the blue values of all acetylated samples are somewhat smaller than those of comparable hydroxypropylated products, and their iodine binding capacity is less extensive.

The obtained data of the carboxymethyl derivatives concerning the iodine binding properties and



4-methyl-2-pentanone 4-*tert*-butylphenol

Fig. 4. Molecular structures of the investigated guest molecules.

the CD-spectroscopical results are equivalent to those of the hydroxypropylated amyloses in the case of the lower substituted products. However, regarding the samples of higher d.s., the observed values of the carboxymethyl amyloses decrease much stronger. The following investigation may give an explanation of this effect.

In contrast to other amylose modifications, carboxymethylation of amylose leads, to a much greater extent, to products substituted at C-3 and C-6 of the anhydroglucose units. This is due to the synthetic conditions because the applied chloroacetic acid is—compared to acetic anhydride and the oxiranes used—much less sensitive against hydrolysis even in alkaline solution. A great amount of it is therefore present in solution for a longer period and promotes substitution at the less reactive positions (C-3 and C-6). We were able to prove this by $^1$H NMR spectroscopy, observing significant signals for the methylene groups of the carboxymethyl substituents.

Substitution of the hydroxy groups at C-3 and C-6 lowers the complex stability because it disturbs the helical conformation of the chain for sterical reasons and prevents intrahelical hydrogen bonding between adjacent turns [9,15]. As a consequence, the observed data for higher substituted carboxymethyl amyloses show less complexing ability.

For the CD-spectroscopic investigations, we dissolved a fixed quantity of modified amyloses in water and added a constant amount of a stock

Table 1
Water solubility and complexing ability of the hydroxyethyl, hydroxypropyl and 1,2-dihydroxypropyl amyloses

| Amylose derivative | m.d.s.[a] | BV[b] | $\lambda_{max}$[c] (nm) | Amount of complexed iodine (mg/g) | Water solubility of amylose | Solubility of fenchone[d] (g/L) | CD (mdeg)[e] | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 4-methyl-2-pentanone | 4-*tert*-butylphenol |
| Unmodified | 0[f] | 12.2 | 640 | 182 | insoluble | complex insoluble | complex insoluble | complex insoluble |
| 2-Hydroxypropyl | 0.04[f] | 11.4 | 620 | 164 | insoluble | complex insoluble | −1.45 | n.d. |
| | 0.06 | 11.2 | 620 | 159 | 5% warm soluble | complex insoluble | −1.45 | −2.30 |
| | 0.13 | 6.8 | 600 | 98 | 5% warm soluble | 1% 3.42 g | −1.40 | n.d. |
| | 0.19 | 3.4 | 570 | 51 | 5% warm soluble | 1% 3.63 g 5% 4.38 g | −1.15 | −1.40 |
| | 0.31 | 0.6 | 530 | 11 | 5% cold in 2 h | 1% 3.66 g 5% 4.42 g | −1.10 | −1.10 |
| | 0.48 | n.d.[g] | n.d.[g] | n.d.[g] | 5% cold in 2 h | 1% 3.34 g | −0.80 | n.d. |
| | 0.72 | 0.1 | n.d. | n.d. | 5% cold in 1 h | 1% 3.37 g | −0.70 | −0.60 |
| | 1.37 | n.d. | n.d. | n.d. | 5% cold in 30 min | 1% 3.39 g | −0.35 | −0.55 |
| 1,2-Dihydroxypropyl | 0.06 | 11.2 | 620 | 162 | 5% warm soluble | complex insoluble | −1.50 | −2.05 |
| | 0.18 | 4.6 | 590 | 75 | 5% warm soluble | 1% 3.51 g 5% 4.49 g | −1.15 | −1.35 |
| | 0.30 | 0.9 | 560 | 14 | 5% cold in 1 h | 1% 3.55 g 5% 4.52 g | −0.85 | −1.00 |
| 1-Hydroxyethyl | 0.15 | 5.6 | 595 | 77 | 5% warm soluble | 1% 3.52 g | −1.20 | −1.50 |
| | 0.50 | 0.2 | n.d. | n.d. | 5% cold in 2 h | 1% 3.30 g | −0.65 | −0.75 |

[a]m.d.s.: average molar degree of substitution, number of moles of alkylene oxide combined per anhydroglucose unit.
[b]BV: blue value (explanation given in text).
[c]$\lambda_{max}$: maximum of absorption of the amylose–iodine-complex.
[d]Solubility of fenchone: determined solubility of fenchone in the mentioned amylose solns. These data consist of the complexed and the dissolved portion of fenchone (2.31 g/L).
[e]CD (mdeg): maximum amplitude of the CD effect in a 2% w/w aqueous amylose soln containing a constant amount of the nominated guest molecule.
[f]These samples were dissolved in 1 M NaOH and neutralized with HCl.
[g]n.d.: not determined.

Table 2
Water solubility and complexing behavior of the acetyl and carboxymethyl amyloses

| Amylose derivative | d.s.[a] | BV | $\lambda_{max}$ (nm) | Amount of complexed iodine (mg/g) | Water solubility of amylose | Solubility of fenchone (g/L) | CD (mdeg) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 4-methyl-2-pentanone | 4-*tert*-butylphenol |
| Acetyl | 0.06 | 8.9 | 600 | 122 | 5% warm soluble | complex insoluble | n.d. | n.d. |
| | 0.14 | 4.0 | 585 | 58 | 5% warm soluble | complex insoluble | −1.76 | −1.76 |
| | 0.16 | 3.9 | 580 | 55 | 5% warm soluble | 1% 3.28 g[b] 5% insoluble | −1.59 | −1.59 |
| | 0.26 | 0.9 | 540 | 13 | 5% warm soluble | 1% 2.30 g 5% 2.94 g | n.d. | n.d. |
| | 0.43 | n.d. | n.d. | n.d. | 5% cold in 2 h | 1% 2.30 g 5% 2.63 g | −1.49 | −1.46 |
| | 0.61 | n.d. | n.d. | n.d. | 5% cold in 1 h | 1% 2.30 g 5% 2.47 g | −1.46 | −1.39 |
| Carboxymethyl[c] | 0.09 | 11.2 | 620 | 158 | 5% warm soluble | complex insoluble | −1.40 | −2.20 |
| | 0.20 | 2.2 | 570 | 34 | 5% warm soluble | 1% 3.55 g 5% insoluble | n.d. | n.d. |
| | 0.23 | 1.8 | 565 | 27 | 5% cold in 2 h | 1% 3.44 g 5% 3.69 g | −0.60 | −1.50 |
| | 0.28 | 0.4 | 530 | 7 | 5% cold in 1 h | 1% 3.25 g 5% 3.99 g | n.d. | n.d. |
| | 0.67 | n.d. | n.d. | n.d. | 5% cold in 30 min | 1% 2.84 g 5% 3.67 g | −0.20 | −0.47 |

Explanation of the abbreviations as shown in Table 1.
[a]d.s.: average degree of substitution, number of substituted hydroxyl groups per anhydroglucose unit.
[b]Data determined by the fenchone drop method (see text for explanation).
[c]All samples dissolved in demineralized water without neutralisation.

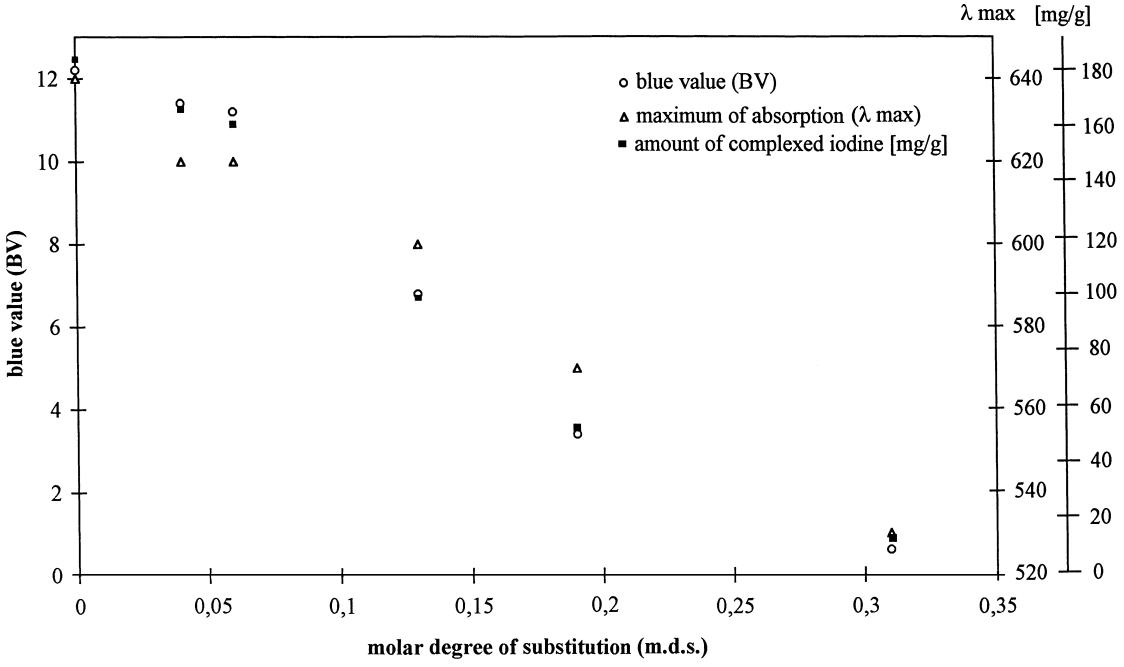



Fig. 5. Dependence of blue value, maximum of absorption of the amylose–iodine-complex and amount of complexed iodine on the degree of substitution of the hydroxypropylated amyloses.

solution containing the desired guest molecule. The solutions were mixed extensively, stored overnight to reach equilibrium and were subsequently examined. The spectra of the 4-methyl-2-pentanone complexes were measured in the wavelength range of 230–350 nm, those of the 4-*tert*-butylphenol complexes between 235 and 350 nm.

As shown in Tables 1 and 2, a negative Cotton effect was observed for all combinations of guest and host molecules. This fact provides evidence for the assumption that both of these guest molecules are complexed in the same conformation regardless of the substituent type. This conclusion can be drawn by the fact that CD-spectroscopy very accurately reflects the molecular conformation of an included guest inside the chiral environment of a given host molecule [27–29].

Furthermore, the extinctions of the induced Cotton effects become continually smaller with increasing degree of substitution of the investigated amylose derivatives which, in agreement with the preceding examinations, confirms the decreasing complexing ability as shown in Fig. 6 for 4-*tert*-butylphenol complexes.

In contrast to this, when comparing complexes of the same guest molecule with different amylose derivatives of analogous d.s., we almost observed comparably sized Cotton effects with both of the guests. On the one hand, these results confirm the equivalent complexing ability of the investigated modified amyloses, on the other hand, it demonstrates their similar ability of enlarging the helical structure up to seven anhydroglucose units per turn.

But this similarity is not true for the acetylated amyloses. Remarkably, the observed Cotton effects, especially with 4-methyl-2-pentanone as guest molecule, are distinctly enhanced when acetylated amyloses are used as host molecules. In this case, the interactions of the organic guests with the amylose helix are obviously intensified. However, it is not clear whether the molar extinction of these complexes is higher than those of the hydroxypropylated amyloses or the complexed amount of 4-methyl-2-pentanone is increased.

Fig. 7 shows the corresponding CD spectra of 2 samples of hydroxypropyl, 1,2-dihydroxypropyl, and acetyl amylose complexes of 4-methyl-2-pentanone possessing the same molar degree of substitution.

As expected, the observed Cotton effects decrease also with increasing degree of substitution. By outlining the complexation behavior of the acetylated amyloses, it should be taken into account that the amount of bound iodine is lower than with hydroxypropylated amyloses.

The use of 4-*tert*-butylphenol as guest molecule induces a helix with seven glucose units per turn. We recognized relatively small Cotton effects for the samples of lower d.s., whereas the higher substituted acetyl amyloses led to much more intense effects compared to the hydroxypropylated ones. Here the decreasing effect of the CD amplitude with increasing d.s. is clearly smaller than for all the other compounds. Specific effects with organic guest molecules occur therefore at higher degree of substitution of acetylated amyloses.

To obtain some quantitative results on the amount of included organic guest molecules, we examined the complexing behavior for the amylose-fenchone complex, because fenchone forms very stable complexes with amylose and its derivatives. To obtain soluble fenchone complexes, we had to employ modified amyloses with a minimum

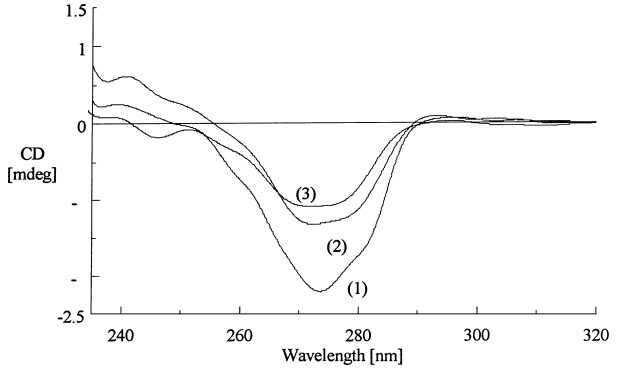



Fig. 6. CD spectra of 4-*tert*-butylphenol complexes with hydroxypropylated amyloses of different degree of substitution. (1) m.d.s. = 0.06, (2) m.d.s. = 0.19, (3) m.d.s. = 0.31. conc.(amylase) = 2% w/w; conc(4-text-butylphenol) = 1.0 $10^{-3}$ mol/l.

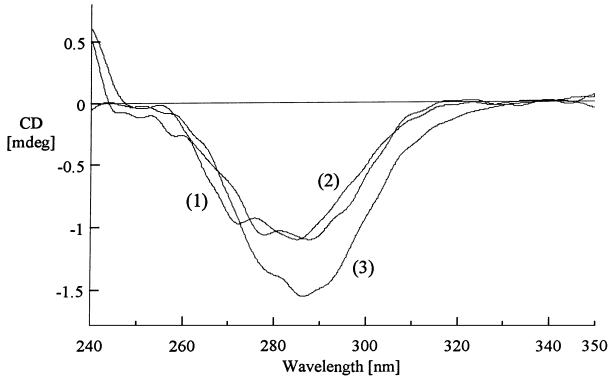



Fig. 7. CD spectra of 4-methyl-2-pentanone complexes with different amylose derivatives of resembling degree of substitution. (1) hydroxypropyl, (2) 1,2-dihydroxypropyl, (3) acetyl.

molar degree of substitution of 0.13 in the case of the hydroxypropyl, hydroxyethyl and 1,2-dihydroxypropyl amyloses. For acetylated amyloses, a minimum degree of substitution of 0.16 and for the carboxymethyl derivatives a d.s. $\geq$0.20 was necessary to get stable complexes in solution. The complexes of lower substituted amyloses are not soluble and retrograde soon.

When 1% w/w complex solutions of the hydroxypropylated amyloses are compared, a maximum in complexing ability for amyloses of m.d.s. 0.20–0.30 is evident. Higher substituted derivatives bind less fenchone. Unexpectedly, only a somewhat higher portion of the ketone was complexed in the 5% w/w solns. Although the amount of host molecules for complexation increases 5-fold, only 60–80% more fenchone is included. A possible explanation for this observation is the intermolecular steric hindrance of the amylose helices in more concentrated polymer solutions.

The complexing ability of the hydroxyethyl and 1,2-dihydroxypropyl amyloses is, in agreement with the results of iodine complexation, equivalent to the comparable hydroxypropylated samples. Differences are within the precision of measurement.

When the experimental data for the acetylated derivatives are compared to those presented in Table 1, it is generally noticed that the complexing ability is distinctly lower. Considering the 1% w/w amylose solns, the highest amount of the guest molecule was complexed with an amylose of d.s. 0.16. With less substituted samples we only obtained insoluble complexes and, by using 1% w/w solutions of higher substituted acetyl amyloses, we surprisingly could not determine any increase in fenchone solubility. Only in 5% w/w solutions we measured a slight increase compared to the 1% w/w solutions, when samples of d.s. 0.26 or above were employed. Less substituted products resulted in insoluble complexes again. Since the quantity of complexed fenchone is evidently lower for all investigated samples compared to the hydroxypropylated ones, acetylation does not seem to be a favorable method for synthesizing water-soluble amylose derivatives in order to obtain inclusion complexes with organic compounds.

The relatively poor water solubility of the carboxymethyl amylose-fenchone complexes is unexpected. We obtained stable solutions only with applying products of d.s. 0.20 or higher. Hence, it is assumed that the carboxyl groups have a rather insignificant influence on the solubility of the samples. The examined fenchone concentrations are in the same range as for the hydroxypropylated substances of equivalent m.d.s.. The maximum amount of complexed fenchone is achieved by using the least substituted product. According to the CD-spectroscopical results, the complexing ability decreases continuously with increasing d.s.. Remarkably, the 5% w/w complex solutions contain only slightly more complexed fenchone as 1% w/w samples. Only about 20% of the available capacity of the amylose helix is used for complexation (data based on a helical conformation assuming 6 AGU per turn), whereas in 1% w/w complex solns up to 85% of the amylose helices will be utilized. Nevertheless, the increase of fenchone concentration with increasing amylose content is higher when the degree of substitution is raised. This fact provides evidence of interactions between the carboxyl groups of the substituents and the guest molecules occurring in higher concentrated solutions.

The pH dependence is important for an entire characterization of the solubility and complexing ability of carboxymethyl amyloses. Table 3 reveals the results for two samples of carboxymethylated amyloses of different d.s. in acidic, basic and neutral solution.

Table 3
pH dependence on the water solubility and complexing ability of the carboxymethyl amyloses

| Amylose derivative | d.s. | pH | Water solubility of amylose | Solubility of fenchone (g/L) | CD (mdeg) 4-*tert*-butylphenol |
|---|---|---|---|---|---|
| Carboxymethyl | 0.23 | 2.3 | 5% cold in 2 h | 1% 3.44 g<br>5% 3.69 g | −1.50 |
| | | 7.0 | 5% cold in 1 h | 1% 3.38 g<br>5% 3.63 g | −1.48 |
| | | 9.0 | 5% cold in 1 h | 1% 3.10 g<br>5% 3.61 g | −1.40 |
| | 0.29 | 2.3 | 5% cold in 1 h | 1% 3.36 g | −1.39 |
| | | 7.0 | 5% cold in 45 min | 1% 2.83 g | −1.30 |
| | | 9.0 | 5% cold in 45 min | 1% 2.85 g | −1.13 |

for explanation of the abbreviations, see Table 1.

The acidic solution at pH 2.3 was prepared by dissolving the freeze-dried sample in demineralized water. Addition of 1 M NaOH and buffer solutions of pH 7.0 and 9.0 yields a neutral and a basic sample. To prevent decomposition of the polysaccharide chains by cleavage of the glycosidic linkage, we did not apply solutions of even higher or lower pH.

Unexpectedly, the influence of the pH of the solution on the evaluated attributes is relatively low. Although the carboxyl groups of the substituents are completely protonated in acidic solution, whereas they exist as carboxylate ions in alkaline medium and hence attract a high degree of counterions, we did not observe considerable differences in complexation.

As can be seen from Table 3, the solubility of the carboxymethyl amyloses in water was found to be reduced in acidic solutions, whereas these samples achieved the highest amount of complexed guest molecules. These observations can be explained with the assumption that the protonated carboxyl groups will disturb the amylose's helical conformation to a lesser extent due to its lower steric hindrance.

In the case of the sample with d.s. of 0.23 we also investigated 5% w/w solutions in order to determine the dependence of pH on fenchone concentration. While the increase of complexed fenchone from 1% w/w to 5% w/w solutions is equivalent in acidic and in neutral medium, it is remarkably enhanced in basic solution. Here interactions of the carboxylate with the organic guest molecule may be responsible.

## 3. Experimental

Potato amylose was supplied by Avebe (The Netherlands). Amylose was reprecipitated twice with cyclohexanone for purification. Propylene oxide, 2,3-epoxy-1-propanol, chloroacetic acid and iodine were commercially available products from Merck (Darmstadt, Germany). Ethylene oxide was obtained from Messer-Griesheim (Düsseldorf, Germany), fenchone from Fluka (Buchs, Switzerland). Acetic anhydride was received from Janssen (Geel, Belgium). 4-*tert*-Butylphenol and 4-methyl-2-pentanone were purchased from Aldrich (Steinheim, Germany). 4-*tert*-Butylphenol was sublimed before use. Hydroxypropylation of amylose was performed as described elsewhere [4].

*Hydroxyethylation*.—The derivatisation was carried out at 2 °C based upon a method elaborated by Banks et al. [30]: Defined amounts of liquid ethylene oxide were added to a 2% w/w soln of amylose in 1 M NaOH under $N_2$ using a Hamilton syringe. The soln was stirred for 15 min, and stored overnight at room temperature. Afterwards, it was neutralized with HCl and added to twice its volume in 96% EtOH. The precipitated amylose was filtered off, washed with EtOH and dissolved again in demineralized water. The soln was filtered and once more precipitated in EtOH. After filtration, the product was washed with EtOH and acetone and dried in vacuum.

*Modification of amylose with 2,3-epoxy-1-propanol*.—Amylose (2 g) was dissolved in degassed 1 M NaOH (100 mL) under stirring in $N_2$. 2,3-Epoxy-1-propanol (1.2–6.0 mL) were added, the soln was stirred for 15 min and kept at room temperature overnight. Thereafter it was neutralized with HCl and poured into 96% EtOH (300 mL). After another 20 min, the precipitated amylose was filtered off and purified as described above.

*Acetylation of amylose*.—Amylose (2 g) were dissolved in degassed 1 M NaOH (100 mL) under continuous stirring. Then the pH of the soln was lowered to 9 by addition of HOAc. $Ac_2O$ (0.2–1.5 g) were added under simultaneous addition of NaOH to keep the pH at 8–9. After complete addition, the soln was stirred for another 15 min and neutralized with HOAc. Further purification of the product was carried out as mentioned before.

*Carboxymethylation of amylose*.—Amylose (2 g) were dissolved in degassed 1 M NaOH (50 mL) in $N_2$. A soln (50 mL) containing 36 g of solid NaOH was added to make it 10 M in base. Subsequently, chloroacetic acid (5.3–20.0 g) was added and the soln was stirred for 3–16 h. After neutralization with HCl and filtration through a medium sintered glass funnel it was dialyzed against demineralized water for 3 days. Then the soln was concentrated and freeze dried.

*Preparation of fenchone complexes*.—In a 25 mL flask substituted amylose (0.1–0.5 g) were dissolved in demineralized water to obtain 1–5% solns. After addition of fenchone (1 mL), the solns were shaken for several hours and stored overnight to reach the complexing equilibrium. Subsequently, they were centrifuged and a sample was taken out of the aqueous phase. It was membrane-filtered (regenerated Cellulose of 0.45 $\mu$m, Sartorius) and investi-

gated by UV spectroscopy at 278 nm to determine the absolute amount of solubilized fenchone. The complexed portion of guest molecule can be calculated from the difference between the given value and the water-soluble amount (2.31 g/L).

UV spectroscopic measurements were conducted on a Perkin–Elmer spectrophotometer 554 using a 1 cm cell.

*Investigation of solubilized fenchone by the fenchone drop method.*—Two solns of the less substituted complexes of acetyl and carboxymethyl amylose showed some opalescence, so we were not able to obtain clear solns to investigate them spectroscopically. In this case, we applied a so-called "fenchone drop method" at which a 1% amylose soln was divided in several equivalent portions and treated with different amounts of fenchone. The solns were mixed carefully and stored overnight. Afterwards, we determined exceeding amounts of fenchone at the solns' surfaces by coloring them with azobenzene.

*Circular dichroism spectroscopy.*—In a 10 mL volumetric flask, amylose (0.2 g) were dissolved in approximately 4 mL demineralized water. One millilitre of phosphate buffer (pH 7.0) and 5 mL of a stock soln containing the desired guest molecule were added and the soln filled up to 10 mL. It was kept overnight and investigated by CD spectroscopy. The CD spectra of 4-*tert*-butylphenol were recorded in the wavelength range 235–350 nm, those of 4-methyl-2-pentanone in the range 230–350 nm. 4-*tert*-Butylphenol was used in a stock soln of $1.9\times10^{-3}$ mol/L, the soln of 4-methyl-2-pentanone contained $9.0\times10^{-2}$ mol/L.

The spectra were recorded on a Jasco CD spectropolarimeter J 600 with cuvettes of 1 cm layer thickness.

*$^1H$ NMR spectroscopy.*—Spectra were recorded on a Bruker DRX 500 (500 MHz) and a Varian VXR 300 (300 MHz).

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