Covalent Binding of Glycosyl Residues to Bovine Casein: Effects on Solubility and Viscosity

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Different simple carbohydrates were bound to the N^{ϵ}-aminolysyl residues of bovine casein by reductive alkylation in order to study the effects on the electrophoretic behavior and on the solubility properties of these proteins according to different factors. These factors included modification degree, type and chain length of bound carbohydrate (aldose or ketose), and the effect of pH, ionic strength, and protein concentration on solubility of the modified caseins. The solubility of all modified caseins is increased in the range of their isoelectric points (pH_i), unchanged at neutral and alkaline pHs, and decreased at low pH values. This effect is marked when the degree of modification and the ionic strength are higher. The solubility depends on the type of glycosyl ligand and is much improved when the glycosyl ligand has a higher molecular weight. The viscosity of the glucosylated and especially of the galactosylated casein was found to be increased at high degrees of modification and at a protein concentration of 10% (w/v).

Developments in food technology and evolution of consumer tastes have created a need for proteins endowed with functional properties suitable for new utilization conditions. Also it is often advantageous to increase viscosity and solubility especially if, in the case of denatured proteins or of casein, the pH of use occurs around the isoelectric point (pH 4.6).

Although it is not often employed in food technology, chemical modification of proteins enables an understanding and may allow for prediction of the relations between structure and functional properties. It is already known that phosphorylation and succinylation are a means for increasing the solubility of caseins at acidic pH values (Girerd et al., 1984), because binding of acidic groups increases electrostatic repulsions. Some studies have been carried out on binding of carbohydrates to proteins and on their effects on digestibility or functional properties. A lower digestibility was found for the sugar-derivatized casein (Lee et al., 1979). Such new compounds were found to have a better solubility than bovine casein at acidic pH values (Canton and Mulvihill, 1983). Others have improved solubility and heat stability, e.g. glycosylated β-lactoglobulin (Kitabatake et al., 1985).

In this study, the respective effects of different carbohydrates bound by reductive alkylation on lysyl residues (monosaccharides glucose, fructose, galactose, and mannose; disaccharides maltose and lactose) on solubility are explained, and in each case the combined effects of protein concentration, ionic strength, and degree of modification are discussed. Viscosity measurements were also performed on some glycosylated caseins.

MATERIALS AND METHODS

Materials and Reagents. Reducing carbohydrates and sodium cyanoborohydride were purchased from Merck; 2,4,6-trinitrobenzenesulfonic acid (TNBS) was from Serva. Bovine casein was prepared in the laboratory by isoelectric precipitation, washed three times with distilled water, and then dissolved at pH 7.0. This process was repeated three times.

Chemical Modification of Casein. The glycosylated caseins were prepared according to Lee et al. (1979), using different reaction times to obtain a range of modification degrees. For all reactions the value of the molar ratio carbohydrate/protein was 70 and corresponded to a molar ratio carbohydrate/lysyl residues equal to 6; these values allow a high initial reaction rate with a good solubility of the reagents. Casein (100 g) and $NaCNBH_3$ (15 g) were dissolved at 40 °C in 1 L of 0.2 M potassium phosphate buffer, pH 8.0, and then mixed with different amounts of carbohydrate. The reaction was carried out at 37 °C for different reaction times. The solution was dialyzed against 0.1 M NaCl solution and three times against distilled water and then freeze-dried. During the dialysis the pH was adjusted to 6 with NaOH in order to prevent protein precipitation.

Determination of Modification Degree. The degree of the reaction was determined by the amount of unreacted ϵ-amino groups of the lysyl residues by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method as described by Habeeb (1966) and Kakade and Liener (1969). The ϵ-TNP derivatives absorb at 335 nm and correspond to the unsubstituted and free ϵ-amino groups of lysyl residues. The degree of chemical coupling is obtained by

$$\text{degree of glycosylation (\%)} = \frac{(A_c - A_a)}{A_c} \times 100$$

where A_c = absorbance of unmodified casein solution (control) and A_a = absorbance of modified casein solution (assay).

This degree of glycosylation represents the percentage of glycosylated lysyl residues. The casein concentration was determined by the Lowry method (1951) with use of control casein as a standard.

Electrophoresis. To assess the effect of the modification on the electrophoretic pattern, polyacrylamide gel electrophoresis (acrylamide 7.5%) in dissociating medium (8 M urea) in the presence of 2-mercaptoethanol was performed following the method of Maurer (1971) confirmed by Ng-Kwai-Hang and Kroeker (1984).

Assessment of Casein Solubility. The solubility index of control and glycosylated caseins was assessed by the following method. In order to obtain casein dispersions at different protein concentrations, ionic strengths, and pH values, each freeze-dried casein was dissolved in distilled water at 25 °C. The required amount of NaCl solution was then added and pH adjusted with 0.1 N HCl or 0.1 N NaOH, and the volume was completed with distilled water to obtain the desired final concentration. After

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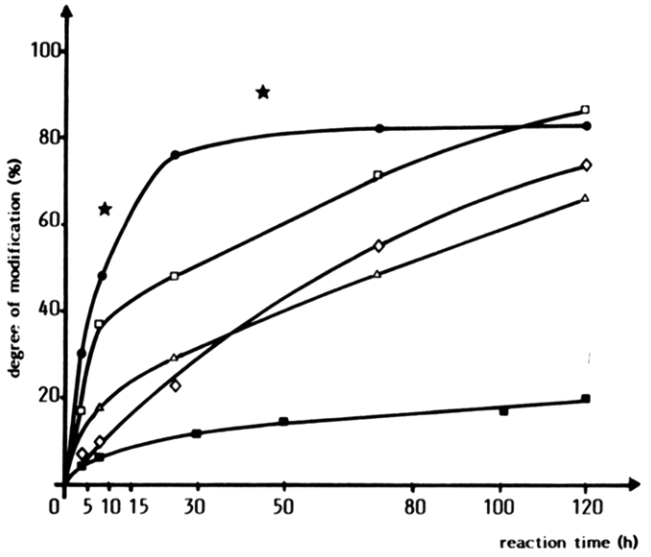

Figure 1. Kinetics of coupling of carbohydrates on casein: glucose (□), galactose (●), lactose (◊), maltose (△), mannose (★), and fructose (■) in the presence of cyanoborohydride at 37 °C in 0.2 M potassium phosphate buffer, pH 8.0.

1 h of continuous stirring, the pH of the dispersion was accurately measured. An aliquot (5 mL) of each dispersion was centrifuged 20 min at 5000*g* with a swinging bucket rotor at 25 °C. The protein content of each supernatant was determined by the Lowry method (1951). The solubility index was calculated as

$$\text{solubility index (\%)} = \frac{\text{supernatant protein concn (mg/mL)}}{\text{protein concn before centrifgn (mg/mL)}} \times 100$$

The Lowry method was exclusively used since it has been checked to provide values in agreement with those obtained by the Kjeldahl method.

Viscosity Determination. Viscosity measurements were made on a rotational viscometer, Rheomat 30 (Contraves, Zurich), coaxial cylindres (MSO, MSB, or MSC) being chosen according to product viscosity. A Rheoscan 20 programmer was used to plot the flow curve $\tau = f(\dot{\gamma})$ automatically, with τ = shear stress (Pa) and $\dot{\gamma}$ = shear rate (s^{-1}). Measurements were always performed at 20 ± 0.1 °C. Casein samples were dissolved in distilled water, the solutions being adjusted to pH 7.1 with NaOH solutions.

All the casein solutions exhibited a Newtonian behavior, the viscosity (in Pa·s) being given by the slope of the flow curve.

RESULTS AND DISCUSSION

Preparation of Glycosylated Casein and Kinetics of Coupling of Carbohydrates on Casein. The influence of reaction time on the degree of glycosylation is shown in Figure 1. The reaction is slow and not complete in spite of very long reaction times (up to 120 h) and a high carbohydrate/lysyl residue molar ratio (6).

There are great differences in reaction rate: The aldoses (glucose, galactose, mannose) are coupled faster than the only ketose tested (fructose) perhaps because of their higher reducing ability. The reaction rate of the disaccharides (maltose, lactose) is intermediate because, at the same molarity, there are half as many reducing groups than those of monosaccharides. Regarding the degrees of glycosylation obtained after 120 h of reaction, our results are consistent with those found by Lee et al. (1979) for glucose (respectively, 80% and 86 ● 4%) and differ for fructose (20% as compared to 62%) and lactose (73% as compared to 17%). It is difficult to obtain higher degrees of glycosylation because the reagents are already quite concentrated [10% (w/v) for casein, 0.2 M for carbohydrates].

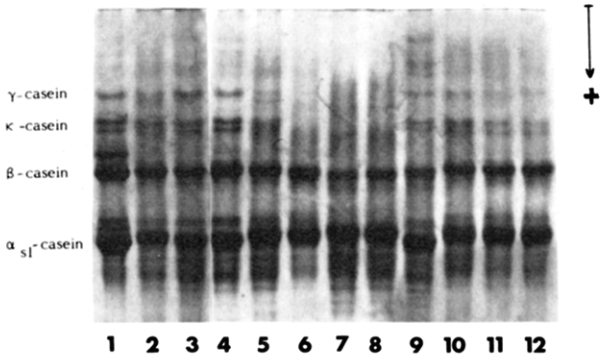

Figure 2. Polyacrylamide gel electrophoresis of glycosylated and control caseins. Conditions: polyacrylamide gel (7.5%), pH 8.6; dissociating medium, 8 M urea; reducing reagent, 2-mercaptoethanol Key: 1, control casein; 2, galactosylated casein (30%); 3, galactosylated casein (47%); 4, glucosylated casein (25%); 5, glucosylated casein (35%); 6, glucosylated casein (49%); 7, glucosylated casein (75.2%); 8, glucosylated casein (92.5%); 9, fructosylated casein (20%); 10, lactosylated casein (30%); 11, lactosylated casein (43%); 12, maltosylated casein (54%).

Electrophoretic Behavior of Glycosylated Caseins. According to Figure 2, the electrophoretic mobility of each casein component is rather unmodified by the glycosylation except for α_{s_1} units, the mobility of which is slightly decreased for the highest levels of modification. However, there is a broadening of the protein bands and a trailing effect. This shows that several molecular forms with varying degrees of modification are present. The staining of protein bands is highly decreased by the glycosylation. This effect can be explained by the increase of solubility of the glycosylated caseins in acidic medium (observed later), which can decrease the fixation of proteins on the gel and the dye binding (Coomassie Blue R250) on the lysyl residues that are the sites of glycosylation. A dye binding test could perhaps be used for quick determination of the level of glycosylation.

Changes in Solubility of Glycosylated Caseins. *Influence of pH and Ionic Strength on Casein Solubility.* The control casein (Figure 3) was found to have minimum solubility at its isoelectric pH (pH_i = 4.6) due to maximum electrostatic attractions; when the ionic strength is increased, the solubility is enhanced in the pH range of the isoelectric point (decrease of the electrostatic attractions and competition of Na^+ with protons causing a salting in effect) and is slightly decreased for the pH far from the pH_i (acidic or basic) (Figure 3). Glycosylation increased the solubility of the modified caseins (Figures 4 and 5) in the pH_i range in the absence of salt, here slightly shifted to the acidic pHs (minimum of solubility at pH 3 for the casein galactosylated at 47%, pH 4 for the casein fructosylated at 20%). If the ionic strength is increased, the solubility is almost unmodified at neutral or alkaline pH values while it is strongly reduced at acidic pH (especially from 0.1 to 0.5 M NaCl); this effect increases with the degree of modification. This can be explained for the modified caseins by a decrease of the p*K* value when the amino groups of lysine are substituted and the net charge of the molecules at this pH is slightly decreased. Similar results were observed with other carbohydrates such as mannose, maltose, and lactose.

Influence of pH and Protein Concentration on the Casein Solubility. The influences of pH and protein

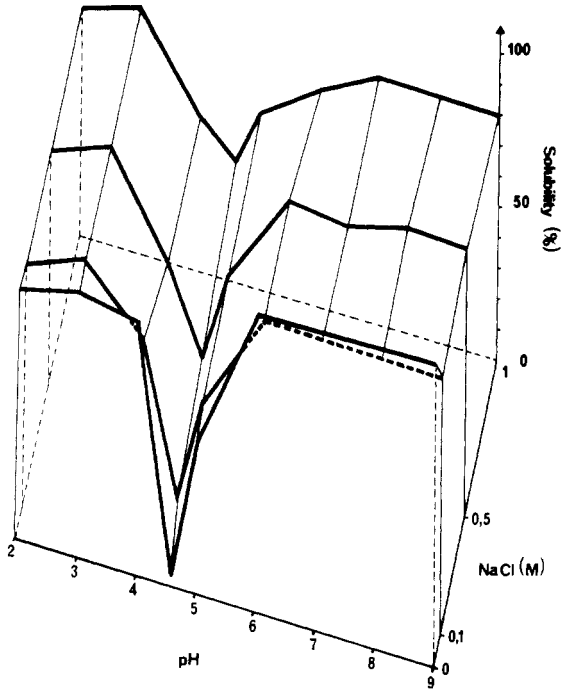

Figure 3. Influence of pH and ionic strength on the solubility of the unmodified casein. Protein concentration 1% (w/v).

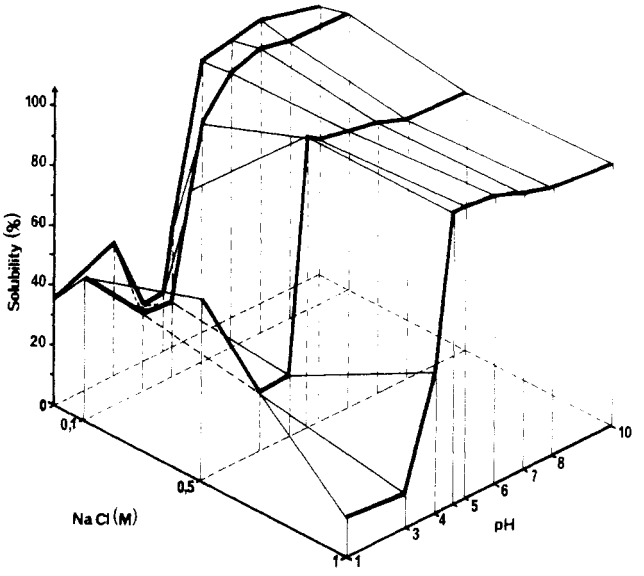

Figure 4. Influence of pH and ionic strength on the solubility of the galactosylated casein. Degree of modification 47%; protein concentration 1% (w/v).

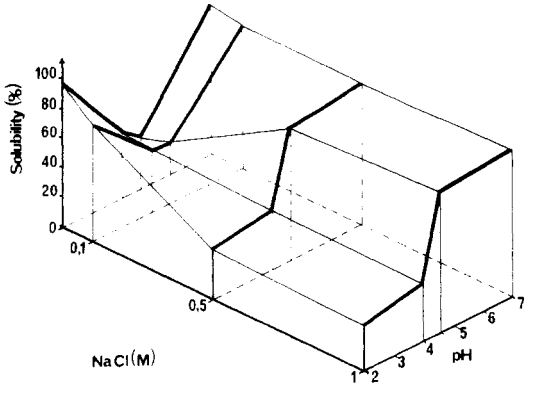

Figure 5. Influence of pH and ionic strength on the solubility of the fructosylated casein. Degree of modification 20%; protein concentration 1% (w/v).

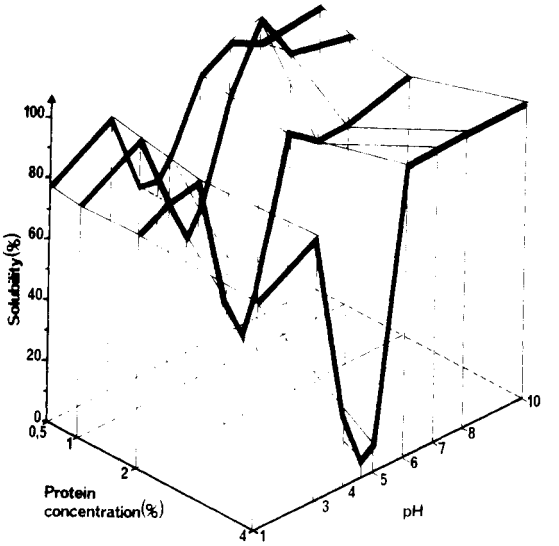

Figure 6. Influence of pH and protein concentration on the solubility of the control casein (NaCl, 0.5 M).

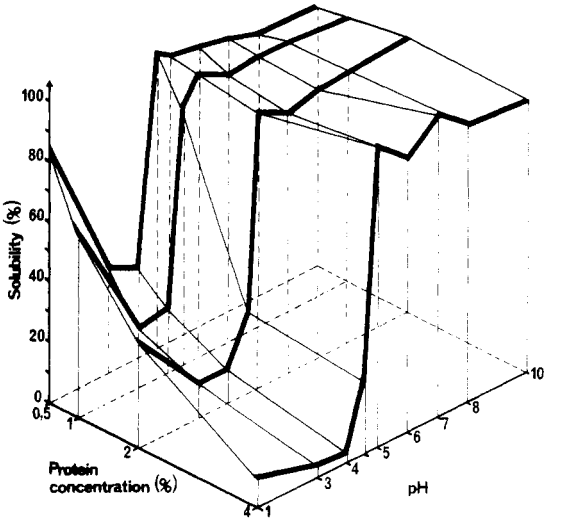

Figure 7. Influence of pH and protein concentration on the solubility of the galactosylated casein. Degree of modification 47% (NaCl, 0 M).

concentration are interdependent (Figures 6 and 7) since the higher the protein concentration, the more the ionic strength changes by pH adjustment. This fact is verified in Figure 6 which shows that especially at acidic pH values (pH range where the positively charged ϵ-NH_2 amino groups increase the solubility of proteins) the percentage of soluble proteins is strongly decreased when protein concentration increases [0.5–4% (w/v)]; this effect is increased by the glycosylation (case of the 47% galactosylated casein), which reduced the positive charge of each amino side chain. These effects of protein concentration were not observed by other authors (Kitabatake et al., 1985) since the protein concentrations in β-lactoglobulin were very low [0.1% (w/v)]. At alkaline pH values, the percentage of solubility was about 90% whatever the concentration and is in agreement with those of Morr et al. (1985).

Influence of the Degree of Modification on the Solubility of Glycosylated Caseins. As seen in Table I, for solutions at pH 4.6, with fixed protein concentration and fixed ionic strength, the higher the degree of glycosylation (galactosylated) the more soluble the protein. Increases in NaCl concentration reverse this trend.

Table I. Influence of the Degree of Modification on the Solubility of the Glycosylated Caseins

degree of modificn, %	solubility of galactosylated casein[a] pH 2.0	pH 4.6	pH 7.0
0 (unmodified casein)	82 ± 4.1	5 ± 0.2	98 ± 4.9
30.0 ± 1.2	71 ± 3.5	17 ± 0.8	97 ± 4.8
46.6 ± 1.9	56 ± 2.8	22 ± 1.1	98 ± 4.5
55.9 ± 2.2	75 ± 3.7	26 ± 1.3	96 ± 4.8
76.1 ± 3.0	97 ± 4.8	39 ± 1.9	95 ● 4.7

[a] Solubility measurements were made by the Lowry method (Lowry et al., 1951). Each value represents the average of two determinations. Protein concentration 1% (w/v); [NaCl] = 0 M.

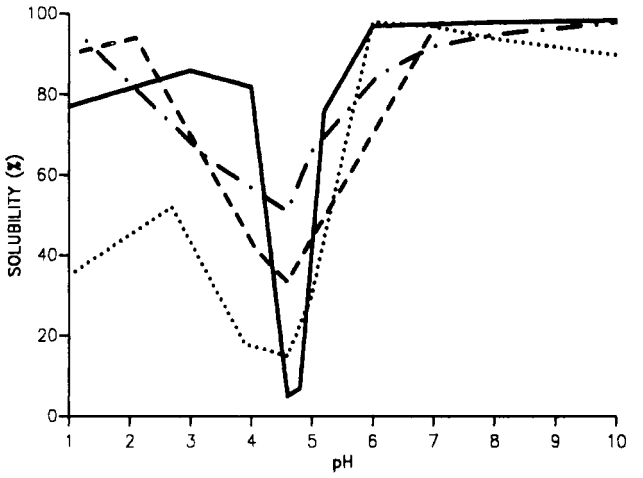

Figure 8. Influence of the kind of bound glycosyl moieties on the solubility of the glycosylated caseins. Protein concentration 1% (NaCl, 0 M). Key: —, control casein; - - -, fructosylated casein, degree of glycosylation 20%; –·–, glucosylated casein, degree of glycosylation 35%; ···, galactosylated casein, degree of glycosylation 35%.

Influence of the Kind of the Bound Carbohydrate. As seen in Figure 8, under the same experimental conditions for solubility studies and degrees of modification at pH 4.5 and at very low ionic strength, the caseins can be graded in order of increasing solubility index: control casein, 5%; galactosylated casein, 16%; fructosylated casein, 35%; glucosylated casein, 52%. Although glucose is only different from the galactose by the position of a hydroxyl (respectively, equatorial and axial positions on carbon 4), it has a higher solubility. As for fructose, its effect on solubility is very important since its degree of modification (20%) is almost twice as small as that for glucose (35%). When the ionic strength is increased (0.5 M NaCl), all modified caseins are more soluble (90%) than the control one (14%) in the pH_i range (pH 4–7), but their behavior is similar and they are only slightly soluble at very acidic pHs. Through the shift of pK values of glycosylated ϵ-amino groups we do not notice an observable change in inflection point for pH precipitation of modified casein.

Influence of the Molecular Weight of the Glycosyl Moiety on Solubility. The solubility of the monosaccharide glycosylated casein was compared with that of the casein modified with the corresponding disaccharide maltose. The same comparison was made between the glucosylated and galactosylated caseins on one hand and the lactosylated casein on the other hand. In these two cases the solubility was much more increased by the disaccharides than by the monosaccharides when fixed at the same level and for the same protein concentration and ionic strength (solubility index of 58% for the maltosylated casein as compared to 26% for the glucosylated casein).

Table II. Viscosity of Glycosylated Casein Solutions at pH 7.1

nature of bound carbohydrate	degree of modificn, %	viscosity, mPa·s 5% (w/v)	10% (w/v)
control	0	4.5	41.0
galactose	32	3.1	47.3
galactose	78	4.5	9716.3
glucose	30	3.2	33.5
glucose	90	4.2	240.0
maltose	27	2.8	17.3
lactose	30	3.8	15.4

At pH 4.6, the solubility was also directly dependent on the number of OH groups bound to casein (four by glucose, seven for the maltose).

Changes in Viscosity of Glycosylated Caseins Solutions. The values given in Table II were calculated from curves of viscosity versus protein concentration over a range of 0–12% (w/v) (Colas et al., 1988). At a protein concentration of 5% (w/v), the viscosities of glycosylated caseins are slightly lower than that of the control casein. At high concentration (10%, w/v), the highly modified caseins (galactose-78 and glucose-90) are 237- and 6-fold, respectively, more viscous than the control one. These results are slightly different from those of Canton and Mulvihill who observed an high increased viscosity only for the glucose-modified caseinate solutions [8% (w/v)] and for a degree of modification of 82%. This behavior reflects high intermolecular interactions between protein chains. It could be explained by the increase of the voluminosity of the molecules due to an increase in the net negative charge and in the steric hindrance after the attachment of sugar residues (Colas et al., 1988).

CONCLUSION

This study confirms some partial results from Lee et al. (1979) concerning the kinetics of glucid attachment to casein by reductive alkylation. It points out the characteristic effects of the structure of each glycosyl ligand (type, molecular weight) on solubility: Canton and Mulvihill (1983) had already observed an increase of solubility by coupling glucose to casein, but they did not describe in detail the relationship of structure to function. The binding of other glycosyl moeities in other ways was also investigated for β-lactoglobulin where the bound sugars were uronic acids (Kitabatake et al., 1985) and maltose and glucosamine (Waniska and Kinsella, 1984).

All these attachments increased solubility and should also appreciably modifiy other functional properties (viscosity and surface properties), but at the present time the latter data remain incomplete. Other results will be published on the effect of covalent attachment of carbohydrates on casein, on other functional properties such as water holding, foaming, and emulsifying properties.

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Changes in Composition of Sunflower Oil Extracted from Achenes of *Sclerotium bataticola* Infected Plants

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Changes in physicochemical components and fatty acid composition of sunflower oil due to *Sclerotium bataticola* infection are reported. In both varieties, cvs. Gloriasol and Romsun HS 301, achenes from infected plants showed a percent reduction in crude fiber content, oil, ash content, and weight of 1000 achenes. However, there were increases in protein content and nitrogen-free extract. Fatty acid composition of oil extracted from achenes of infected plants showed appreciable differences compared to healthy plants. Differences were also observed in cation concentration.

The sunflower (*Helianthus annuus* L.) is one of the most widely grown spring crops in Italy. In 1986, the area under cultivation exceeded 100 000 ha, and oil seed production reached about 1.9 million tons. However, the variety, cultural practices, and environmental conditions, as well as diseases, may adversely affect yield and reduce seed quality. Both fatty acid composition and physical and chemical properties are important for evaluating oil quality. Since genetic factors control oil quality independently of the environment (Putt et al., 1969), it differs considerably from one sunflower variety to another. In addition, diseases such as downy mildew (*Plasmopara helianthi* Novot.) and verticillium wilt (*Verticillium albo-atrum* Reinke and Berth) may modify fatty acid composition and so affect the quality of the oil (Zimmer and Zimmerman, 1972).

The casual agent *Sclerotium bataticola* Taub. [*Macrophomina phaseolina* (Tassi) Goid.], which has a wide host range and grows best at high temperatures, is responsible for charcoal rot, one of the major sunflower diseases in all Italian growing areas (Zazzerini et al., 1985). Attacks by this fungus reduce both seed yield and the oil content of the seeds (Zazzerini et al., 1987).

The present study was undertaken to investigate the effect of *S. bataticola* on the physicochemical components and the fatty acid composition of sunflower achenes.

MATERIALS AND METHODS

Ten healthy and ten *S. bataticola* infected plants were randomly collected from each of the cvs. Gloriasol and Romsum HS 301 at seed development (stage 5.3) (Siddiqui et al., 1975). Both varieties were susceptible to the fungus, but attacks were heavier on Romsun HS 301 (69%) than on Gloriasol (33%). Achenes from both healthy and infected plants were stored for physical and chemical analysis.

The weight of 1000 achenes was determined for each of the six samples. After the achenes had been frozen in liquid nitrogen and ground to meal in an Osterizer mill, the following analyses were carried out.

Moisture of the meal was calculated and used as the basis for estimating dry matter components. A Kjeltec system apparatus (Tecator) was employed for measuring protein. The methods adopted are those recommended in Official Methods of Cereal Analysis (Italian Ministery of Agriculture and Forestry, 1987). After ash content had been calculated, 10 mL of a 50% HCl solution was added to the ash and slowly evaporated to dryness in a water bath. The treatment was repeated, the resulting solution filtered, and the volume adjusted to 100 mL with distilled water. A Pye Unicam SP 9 atomic absorption spectrophotometer was adopted for determining cation concentration (ppm).

Whole seed oil content was measured both with an NMR analyzer and by extraction with diethyl ether using a Soxhtec system (Tecator). The methods used for estimating fatty acid composition are those described in the Gazzetta Ufficiale (1981). Briefly, methyl esters of the fatty acids were prepared by methanolysis and the fatty acid composition of the oil extracted from each sample was analyzed. A 250-μL portion of a 2 N KOH solution in methanol and 5 mL of *n*-hexane were added to 500 mg of oil. The solution was mechanically shaken for 2 min, and phases were separated for GLC analysis. Fatty acid composition of the methyl esters was determined by capillary gas chromatography on a Carlo Erba 4160 gas chromatograph equipped with a split/splitless injector and a flame ionization detector. The column was a fused silica capillary column (30 m × 0.32 mm (i.d.)) coated with a 0.25-μm film SP 2340 (Supelco). Oven temperature and injector and detector temperatures were maintained at 180 and 210 °C, respectively. The flow of helium carrier gas was 1.5 mL/min and the split ratio 1:80.

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