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Identification of the Atopic Allergen in Cow's Milk

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Introduction

Cow's milk contains at least 20 separate protein components which may lead to antibody production in man [28, 29]. In fact several authors reported the occurrence of antibodies to various milk proteins in the sera of apparent healthy individuals [20, 38, 45, 53]. ROTHBERG [45] showed, for example, that 69% of the sera of children of 15 years of age or younger, contained antibodies against bovine serum albumin and 36% contained antibodies to α -lactalbumin. Recently evidence has been presented to show that these antibodies sometimes may play a pathogenic role, especially in some cases of chronic respiratory disease, in gastro-intestinal disorders and in anaphylactic shock. HEINER [30, 32] and HOLLAND [33] described a syndrome characterized by recurrent pneumonia, iron deficiency anemia, pulmonary hemosiderosis and failure to thrive in patients (predominantly children) with multiple precipitins to milk proteins. The symptoms subsided with the elimination of milk and dairy products from the diet and returned when the patients were given cow's milk [30, 33].

Many authors have given detailed accounts of patients with (adult) coeliac disease, ulcerative colitis, regional enteritis and other gastro-intestinal disorders, in the sera of which usually rather high concentrations of precipitating antibodies to milk proteins are demonstrable [31, 35, 50, 54]. It is quite certain that in the majority of the persons with these disorders, precipitating antibodies to milk proteins do not play an etiological role in the elicitation of symptoms [54]. Evidence has been presented to indicate however, that in some cases of gastro-

intestinal disorders antibodies to milk proteins may provoke clinical symptoms or may lead to aggravation of the disorders. Worth mentioning in this respect is the study of WALDMANN [51] which described 6 patients with a syndrome characterized by oedema, anemia, severe hypoproteinemia and growth retardation. He found a correlation between ingestion of milk leading to aggravation of the symptoms and the presence of precipitating antibodies. The symptoms were observed to disappear completely after elimination of milk; the patients were found not susceptible for lactose and wheat gluten. Comparable results were obtained by SEWELL [48], DAVIDSON [22] and GERRARD [24] in some patients with steatorrhoea or showing intractable diarrhoea of otherwise unknown etiology.

In addition, abundant evidence indicates that cow's milk also may lead to clinical symptoms of asthma, rhinitis and atopic dermatitis [1, 17, 24, 25, 26, 52]. These diseases occur in so-called atopic individuals with a predisposition to develop hypersensitivities to various inhalant and food allergens. Atopic phenomena are thought to be mediated by reagin or skin sensitizing antibody, belonging to IgE class of immunoglobulins, whereas it is very likely that in the cases of the above - reported gastro-intestinal and respiratory disorders precipitating antibodies of the IgG class play a role. With regard to cow's milk allergy it seems justifiable therefore to make a distinction between hypersensitivity phenomena in which precipitating antibodies and protein antigens are involved and atopic diseases. In the case of atopic hypersensitivities no correlation will be found between clinical symptoms and, with the commonly used immunological methods, demonstrable antibodies to milk proteins. On the other hand positive (direct or Prausnitz-Küstner) skin reactions may be expected to occur.

This distinction is for example substantiated by the study of GOLDMAN [25, 26]. He found that strong skin reactions to milk and milk fractions only occurred in atopic children who showed clinical symptoms of rhinitis, asthma and atopic dermatitis after challenges with milk. A close correlation was found between the results of strongly positive skin tests to milk protein fractions (β -lactoglobulin, α -lactalbumin, bovine serum albumin and casein) and oral challenge tests with the same proteins. These patients did not have a higher incidence of milk precipitating antibodies than normal, non-allergic persons and atopic individuals not hypersensitive to milk. Of some significance in this respect is also the study of Luz [36]. He found that by heat treatment (13 min, 245°F) the anaphylactogenic activity of milk, as detect-

ed by passive cutaneous anaphylaxis in guinea pigs and Schultz-Dale tests, markedly diminished, whereas the Prausnitz-Küstner activity with reagin sera of milk atopic patients hardly showed a difference.

In spite of the vast amount of work concerning milk hypersensitivity no further detailed attempts have been described on the identification of the allergenic component in milk which is able to elicit wheal and erythema reactions in skin tests and clinical symptoms in atopic individuals. Interestingly, extensive experimental work designed for the identification of atopic inhalant and some food allergens indicated that biological activity is associated with (glyco)proteins with characteristic chemical properties [6, 8, 15, 16, 17, 18]. For example, the molecular weights of the purified allergenic components were found to be of the same order of magnitude; those of the active principles isolated from tomato fruits (20,000-30,000), cod (18,000, ref 2,3) and egg white (31,500) fall in that range. Furthermore the majority of the proteins present in the investigated foodstuffs, although antigenic in man, proved to be not active in skin tests. This suggests that some protein antigens are highly allergenic whereas others apparently are not. It is very likely therefore that in milk hypersensitivity of atopic origin also a specific allergenic component is involved, probably being present in milk in small amounts only.

The object of the present study has been to isolate the 'allergenic' component of milk and to elucidate its nature.

Contact with milk will take place as a rule by means of ingestion of pasteurized or otherwise processed milk formulas. Therefore it is very likely that individuals who are clinically allergic to milk have become sensitized by components present in heated milk preparations. Actually it cannot be excluded that heating influences the allergenic potency and accordingly the skin reactivity of milk, either diminishing or increasing it. Pasteurization for example, has been shown to lead to modification of the proteins accounting for a loss or decrease of the antigenicity of some of these [29, 36, 44, 46]. On the other hand some evidence has been presented to indicate that heating may give rise to an increase of the anaphylactogenic properties of milk [12, 37]. In order to include these effects we decided to start our studies on the identification of the allergenic component(s) in milk with the fractionation of commercially pasteurized milk samples. In comparison, raw (skimmed) milk was used. The fractions obtained were assayed for skin reactivity in atopic individuals showing wheal and erythema reactions after intracutaneous injection of milk.

Materials and Methods

Materials

Fractionation studies were performed with raw milk and commercially available samples of pasteurized milk. Raw milk was obtained from the Control Station for Milk and Dairy Products in Utrecht. It was stored 4–16 h at 4° before use.

β -Lactoglobulin (freeze-dried, lot No. 6930), bovine serum γ -globulin (freeze-dried, lot No. 6399) and α -lactalbumin (lot No. 9887) were purchased from Nutritional Biochemicals Corporation (Cleveland, O.). Bovine serum albumin (trocken reinst) and rabbit anti-sera against bovine serum were obtained from Behringwerke (Marburg-Lahn). The protein samples were found to be heterogeneous with the aid of starch and agar gel electrophoresis; α -lactalbumin contains, for example at least two other protein components. They were used only for preliminary identification of the components on electropherograms.

Greater quantities of β -lactoglobulin, α -lactalbumin and casein were isolated in our laboratory from raw milk. β -Lactoglobulin was prepared and purified after the method of ASCHAFFENBURG [4] and was recrystallized three times. α -lactalbumin was isolated after BLEUMINK [13] and recrystallized by the method of GORDON [27] till a homogeneous preparation was obtained.

Casein was separated from milk by precipitation at pH 4.65. To this end milk was titrated with N HCl to a pH of 4.65. This mixture was allowed to stand for 4 h at 4° and centrifuged (Christ centrifuge, 3500 rpm). The upper layer containing the lipid material and the supernatant solution were discarded, and the sediment encompassing the casein proteins was washed with 0.1 sodium acetate-acetic acid buffer of pH 4.65. The protein mixture was dissolved in water at pH 7 by the slow addition of N NH₄OH. This procedure was repeated twice and the solution subsequently dialyzed against distilled water and lyophilized.

Physicochemical and Analytical Methods

Sedimentation studies were performed with 1% solutions in 1% NaCl in a Spinco Model E centrifuge. Pictures were taken after the rotor had reached full speed (59,780 rpm), temperature 15–17°, angle 50°.

Ultraviolet absorption spectra were determined of 0.05 or 0.1% solutions in 0.01 M, pH 7 phosphate buffer at room temperature against the buffer as a blank, using a Zeiss spectrophotometer type PMQ II equipped with quartz cells of 1 cm light path.

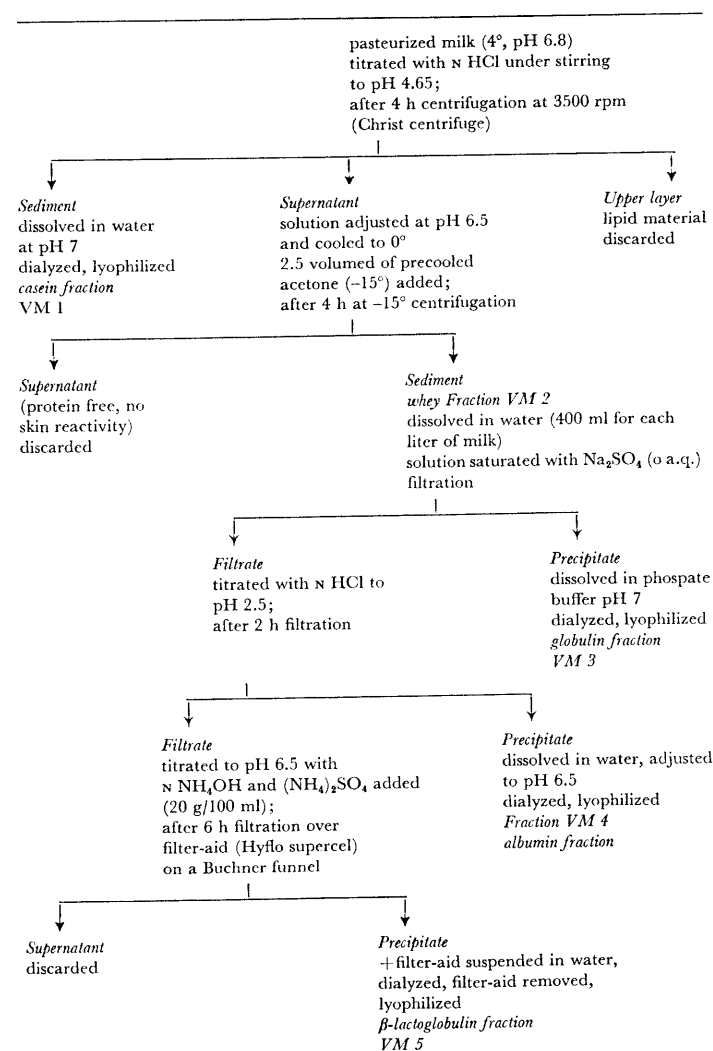
Fluorescence emission spectra were determined of 0.05 or 0.1% solutions under the same conditions as above, using the Zeiss spectrophotometer, equipped with a special fluorescence attachment; excitation energy in this instrument is provided by a constant energy low-pressure mercury arc, from which a beam of monochromatic light of 365 nm wavelength is isolated by means of a filter inserted between the energy source and the cuvettes. The beam of fluorescent light is withdrawn perpendicular to the exciting beam and the spectral distribution is obtained by dispersion of the beam in a quartz monochromator.

Methods of assay for protein nitrogen, hexoses and uronic acids have already been given [15, 18]. Agar- and starch gel electrophoresis were carried out after the methods of WIEME and SMITHIES, as outlined in an earlier paper [18]. Immuno-electrophoresis was done in agar gel at pH 8.6 by the micro-technique of SCHLEIDEGGER [47]; the rabbit antisera to bovine serum were used undiluted, and 5% solutions of the protein fractions in veronal buffer of pH 8.6 were applied to the wells.

Free ϵ -aminogroups (available lysine) were determined by a micro modification of the method of CARPENTER [17, 21].

Proteolytic enzyme digestions were carried out with trypsin (Nutritional Biochemicals Corporation, Cleveland, O.) at pH 8 and 37° under a constant stream of nitrogen in a

Scheme 1. Fractionation of pasteurized milk



pH-state, attached to a Radiometer TTT-1 titrator, equipped with a glass electrode and a magnetic valve; 0.01 M NaOH was added from a micro burette with automatic pH control to a solution containing 25 mg protein in 10 ml of a 0.01 M CaCl₂ solution. After 10 min of preincubation, 20 μ l of a 0.5 percent trypsin solution in 0.001 M HCl was added and the burette was read every 5 min. From the NaOH uptake the number of peptide bonds split per molecule of protein may be calculated.

The monosaccharide composition of the protein fractions was determined after hydrolysis on cation exchange resin (Dowex 50 W $\times$ 8, H⁺-form) in aqueous suspension at 100° for 16 h. The identification method has been described in detail elsewhere [15].

Fractionation Procedures

After some preliminary attempts the procedure, described by ASCHAFFENBURG for the isolation of β -lactoglobulin, was adopted for the fractionation of the milk proteins [4]. This method was slightly modified in such a way that after precipitation of the casein at pH 4.65 the milk whey proteins were separated from the rest of the solution by the addition of 2.5 volumes of pre-cooled acetone. This precipitate was dissolved in water and the proteins were separated in three fractions with the aid of salt fractionation, similar to the method of ASCHAFFENBURG. The fractionation procedure has been given in scheme 1.

Five fractions were obtained, designated as VM 1-VM 5. Pilot experiments had shown that the dialyate of pasteurized and raw milk and the supernatant fraction of the acetone precipitation step did not show allergenic activity in skin tests in atopic individuals with positive skin assays to milk. For these purposes the dialyate and the supernatant solutions were concentrated in a vacuum rotatory evaporator and subsequently lyophilized. In the following experiments these fractions were discarded.

For further identification purposes also the β -lactoglobulin fractions were isolated from pasteurized (VM 6) and raw milk (VM 7) after a slightly different method. In this procedure the casein and globulin fraction were precipitated by the addition of Na₂SO₄ (20 g water-free salt for each 100 ml of milk). After 3 h the mixture was centrifuged. The sediment and the top layer (containing the milk fat) were discarded. Sometimes additional filtration proved necessary in order to obtain a clear (yellow) solution. The α -lactalbumin and β -lactoglobulin fractions were precipitated as outlined in scheme 1, starting with the supernatant solution of fraction VM 3.

In addition the proteose fraction had been isolated from pasteurized milk. To this end pasteurized milk was heated at 95° during 30 min, after which the casein fraction was precipitated at pH 4.65. The mixture was allowed to stand at 4° overnight and the insoluble material was separated from the solution by centrifugation. Subsequently the solution was brought at pH 6.5 and ammonium sulphate was added under stirring to 100% saturation. The precipitate that formed was filtered off, dissolved in phosphate buffer of pH 7, dialyzed and lyophilized (Fraction VM 8). The yield was about 800 mg/l of milk. The protein composition of the proteose fraction is comparable to the milk whey constituents which remain soluble (although protein modification occurred to some extent) after household handling of pasteurized milk.

Skin test Assays

The various fractions obtained were tested for skin reactivity in 9 atopic patients with strong intracutaneous immediate reactions to a commercial milk extract (Bencard). These patients showed clinical symptoms of atopy, all of them had atopic dermatitis and some also rhinitis or asthma. In addition to positive skin reactions to cow's milk they also reacted to a number of inhalant allergens and food extracts. On other grounds also - positive family history of atopy, blood eosinophilia - an atopic hypersensitivity seems evident.

The data of these persons have been collected in table 1.

Table 1. Clinical data of patients tested for skin reactivity with various milk fractions

Patient	Age	Clinical symptoms of atopy	Blood eosinophils number per mm ³	Family history of atopy ¹	Inhalant allergens	Positive skin reactions to Food allergens ²
1	15	atopic dermatitis	308	?	human dandruff, house dust, moulds, feathers	egg, milk
2	32	rhinitis, asthma	?	+	human dandruff, house dust, pollen, moulds, feathers	egg, milk
3	41	atopic dermatitis	?	+	human dandruff, house dust, feathers	milk
4	3	asthma, rhinitis, atopic dermatitis	33	+	human dandruff, house dust, moulds	egg, milk
5	25	asthma	264	—	human dandruff, house dust, pollen	egg, milk, flour, tomatoes
6	15	atopic dermatitis	627	+	feathers, human dandruff, house dust, moulds, feathers	fish, spinach, pork
7	39	asthma (atopic dermatitis)	374	+	human dandruff, house dust, pollen	egg, milk
8	18	atopic dermatitis	286	+	moulds	egg, milk, vegetables
		rhinitis			human dandruff, house dust, pollen	milk
9	45	atopic dermatitis	360	+	moulds, feathers, human dandruff, house dust, moulds, feathers, kapok	egg, milk

¹ + means that atopic diseases were observed in family members also.

² Where the presence of positive skin reactions to fish, shell fish, fruits, flour and vegetables is indicated the patients had positive skin reactions to several kinds of fish, fruits etc.

Skin reactions have been observed and graded as before [15, 17]. In order to enable a comparison of the relative potency of the fractions, on a semi-quantitative basis the activity was expressed in units per mg dry weight of material. The calculation of the biological activities has been described in detail elsewhere [15, 17].

Results

Fractionation of Milk Proteins

Pasteurized milk was submitted to fractionation experiments as outlined in the methods section and the various fractions obtained were tested for skin reactivity in 5 atopic patients with positive skin reactions to milk. In table II the yields, the protein composition and the biological activities of the fractions have been collected.

In the first fractionation step the milk proteins were separated into the casein fraction VM 1, containing more than 80% of the protein material, and the whey fraction VM 2. Of these fractions, VM 2 was observed to contain the active component, and the casein fraction showed only residual activity. Subsequently the whey fraction was

Table II. Yields, protein composition and skin reactivities of fractions obtained from pasteurized milk¹

Fraction	Yield ²	Protein composition ³	Skin reactivity ⁴ [units/mg]
VM 1	32.5 g	casein and traces of whey proteins	<100
VM 2	2.5 g	whey proteins	500
VM 3	750 mg	(eu)globulines, serum albumin, ⁵ α -lactalbumin ⁵ , β -lactoglobulin ⁵	<100
VM 4	850 mg	serum albumin, α -lactalbumin, two not further identified components and small amounts of β -lactoglobulin	500
VM 5	500 mg	β -lactoglobulin	2500
VM 8	800 mg	proteose fraction ⁵	<100

¹ Commercially pasteurized milk, for fractionation see scheme 1.

² Yield per liter of milk.

³ As identified with the aid of agar-and starchgel electrophoresis [18] and immuno-electrophoresis [47].

⁴ Average skin reactivities of 5 atopic patients with a positive skin test to milk (table I and ref. 15).

⁵ (Partly) denaturated components.

separated into three fractions. In VM 3 electrophoresis showed the presence of heterogeneous (denatured) protein material, of euglobulines, bovine serum γ -globulines in addition to minor amounts of bovine serum albumin, α -lactalbumin and β -lactoglobulin.

Fraction VM 4 contained the bulk of the bovine serum albumin and α -lactalbumin protein constituents, whereas in VM 5 only β -lactoglobulin seemed to be present.

The protein composition of the various fractions differed, both quantitatively and qualitatively, from that obtained when raw milk is subjected to the same fractionation procedure. This may be attributed partly to modification and denaturation of the protein components during pasteurization. Heat modification of the proteins will alter the physicochemical properties and accordingly the behaviour of (some of) the proteins during salt fractionation. Furthermore, protein denaturation may have occurred during acetone precipitation.

Of the three whey protein fractions VM 3 appeared to show minimal activity, whereas the activity of VM 4 appeared equal to VM 2. Fraction VM 5 was found to have an average skin reactivity of 2,500 units per mg dry weight material, that is five times higher than that of VM 2.

Interestingly, the proteose fraction VM 8, obtained from pasteurized milk heated during 30 min at 95°, proved to show residual activity as compared with the whey fraction VM 2. Fraction VM 8 contains the whey proteins, which are soluble after the heating of milk, a treatment that is comparable to commonly used household handling. This finding indicates that during the boiling of pasteurized milk the allergenic activity may diminish markedly.

Further Characterization of the Active Component

In the most active fraction, VM 5, agar gel electrophoresis (fig. 1b) revealed the presence of β -lactoglobulin A and B (genetic variants). On immuno-electropherograms, using a rabbit antiserum to bovine serum, sometimes a faint precipitation arc in the serum albumin region was visible (fig. 1a). In fraction VM 4, accounting for 30% of the total activity of the whey proteins, bovine serum albumin and α -lactalbumin could be detected as major constituents. This fraction was also found to contain β -lactoglobulin, traces of bovine serum components and two unidentified proteins.



Fig. 1. Electropherograms of milk fractions.

(a) Immuno electrophoretic pictures of fractions VM 1, 2, 3, 4, 5 and 8 against rabbit antisera to bovine serum components. Left: agar gel electrophoresis of purified bovine serum albumin (BSA) and β -lactoglobulin (LG). (cas=purified casein, whey=milk whey proteins of raw milk.) Anode side at the top of the pictures.

(b) Agar gel electrophoretic pictures of fractions VM 1, 2, 3, 4, 5, 7 and 8 in comparison with

For further identification purposes, skin reactions were done with the major components present in VM 4 and 5 in three of the five atopic patients which showed strong reactions to VM 5. Whereas less than $1 \mu\text{g}$ of VM 5 was found to elicit strong positive intracutaneous reactions, no positive responses were observed after injection of 1 and $10 \mu\text{g}$ of purified bovine serum albumin, α -lactalbumin and recrystallized β -lactoglobulin.

This finding favours the assumption that the major protein components of VM 4 and 5 (in native form) are not responsible for the observed skin reactivities. Accordingly it is most likely that some other protein component present both in VM 4 and 5 elicited the positive skin reactions and that by the fractionation procedure followed, the active component has been concentrated in VM 5, while lower amounts of the same component have been precipitated in VM 4. Therefore we directed our attention in the rest of our studies to the β -lactoglobulin fraction VM 5.

The β -lactoglobulin fraction VM 6 (yield 1.8 g/l of milk) isolated from pasteurized milk by a slightly different procedure from above (compare Methods section) appeared to have an activity something lower than that of VM 5. The skin reactivity of a similar fraction isolated from raw cow's milk (VM 7, yield 1.8 g/milk) was found to be about 500 units/mg dry weight.

The skin reactivities of the various fractions, containing the β -lactoglobulin proteins, have been collected in table III.

In addition these fractions were subjected to chemical analysis and the physico-chemical characteristics of the protein components were established with the aid of electrophoresis and ultracentrifuge methods. Also the ultraviolet absorption and fluorescence spectra were determined. The sedimentation pictures of VM 5, 6 and 7 were found to be similar to that of crystalline β -lactoglobulin; the sedimentation coefficients were calculated at $S_{20} = 3.2$ (1 % protein in 1 % NaCl solution, pH 5.5).

Agar- and starch gel electropherograms (fig. 1b) of VM 5, 6 and 7 showed only the two protein bands of β -lactoglobulin A and B; the

those of purified casein (cas), β -lactoglobulin (LG) and α -lactalbumin (LA). Stain: Naphtol Blue Black (for protein).

(c) Agar gel electrophoretic pictures of fractions VM 5 and C (modified β -lactoglobulin). Stain: Periodate-fuchsin Schiff (for carbohydrate).

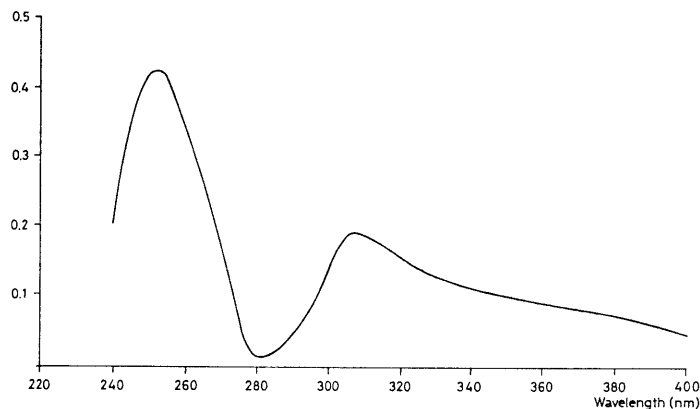


Fig. 2. Difference curve of ultraviolet spectra of VM 5 and crystalline β -lactoglobulin. Spectra of 0.2% solutions of the proteins in 0.01 M, pH 7 phosphate buffer.

electrophoretic pictures indicate, however, some polydispersity. With the aid of immuno electrophoresis in VM 6 and 7 no bovine serum components could be detected. On the other hand remarkable differences were observed in studying the ultraviolet absorption and fluorescence emission characteristics of the various protein fractions.

The extinction coefficients ($E_{1\%}^{1\text{cm}}$) at 305 nm of solutions of VM 5, 6 and 7 proved to be higher than that of crystalline β -lactoglobulin, while in contrast the absorptions at 280 nm were found to be something lower. At wavelengths over 400 nm the protein samples showed, even in higher concentrations, no appreciable absorption. Since the milk protein lactotransferrin has an additional absorption peak at 450–470 nm and lactoperoxidase at 412 nm [23] the possibility may be eliminated that these proteins are responsible for the observed differences in UV absorption.

Difference curves, obtained by subtracting the absorption curve of crystalline β -lactoglobulin from that of VM 5, 6 or 7, revealed peaks at 250 and 305 nm, indicating the presence in these preparations (fig. 2) of additional chromophores. Similar characteristic maxima have been observed in the difference curves constructed for purified atopic allergens from widely different sources [6, 16]. Extensive investigations have indicated that the observed absorption peaks may

be attributed to the incorporation of N-substituted-1-amino-1-deoxy-2-ketose residues within the protein molecules of atopic allergens [5, 6]. These groups may be produced artificially by non-enzymatic (Maillard) reactions between reducing sugars and proteins leading to the formation of 1-deoxy-2-ketose groups and their derivatives linked to the ϵ -amino groups of lysine (of proteins) through carbon atom 1 [5, 6]. These groups are also responsible for the characteristic fluorescence of solutions of atopic allergens and synthetic 1-deoxy-hexulose-proteins [8]. Likewise, solutions of VM 5, 6 and 7 were found to emit blue fluorescence upon irradiation with ultraviolet light of 365 nm wavelength (table III).

These findings strongly suggest the occurrence of 1-deoxy-hexulose-protein constituents in the active β -lactoglobulin preparations, formed artificially by non-enzymatic browning reactions between protein material and lactose during storage and processing of milk.

The incorporation of lactulose residues within the protein material of the fractions VM 5, 6 and 7 is also confirmed by the increased bound hexose contents and by the observation that the β -lactoglobulin bands of the skin reactive fractions could be clearly made visible with the carbohydrate stain, periodate-Schiff (fig. 1c). Moreover after hydrolysis with Dowex 50 on chromatograms only galactose could be detected, whereas in the case of adsorption of lactose both glucose and galactose would have been detected on the paper chromatograms [14, 19].

It is significant that the data recorded in table III gave evidence to indicate that there is a correlation between the degree of browning, as determined by the fluorescence intensity and the ultraviolet absorption at 305 nm, and the skin reactivities of the various fractions.

Thus there are two possible explanations for the observed discrepancy between the skin reactivities of the isolated fractions VM 5, 6 and 7 and purified β -lactoglobulin:

(1) a component present in minor amounts in the fractions accounts for the observed skin reactivities,

or (2) by browning reactions modified β -lactoglobulin molecules elicit the wheal and erythema phenomena in atopic individuals with positive intracutaneous reactions to cow's milk.

In order to evaluate the latter possibility, model experiments were performed, using mixtures of purified β -lactoglobulin and lactose, so that the influence of the incorporation of N-glycosidically bound lactose on the skin reactivity of the protein could be studied.

Table III. Analytical data and skin reactivities of isolated β -lactoglobulin samples

Preparation	Skin reactivity ¹ [units/mg]	Hexose content ² [%]	Absorption [E] % 280 nm	Absorption [E] % 305 nm	E 280 nm/ E 305 nm	Fluorescence ³ [Int. 450 nm]
VM 5	2500	1.1	9.20	1.95	4.8	92.5
VM 6	1500	1.0	9.20	1.75	5.3	47.0
VM 7	500	0.55	9.50	0.70	13.5	39.5
crystalline β -lacto- globulin	100	0.15	9.50	0.70	13.5	20.0
ibidem						
3 times recrystallized	<100	0.1	9.60	0.60	16.0	7.2

¹ Skin reactivity in units/mg dry weight, average of five atopic patients with positive skin tests to milk (table I).

² Expressed as % galactose [15].

³ Fluorescence intensity in arbitrary units (amplified 250 times), of 0.1% solutions in 0.01 M, pH 7 phosphate buffer.

Model Experiments with Lactose- β -Lactoglobulin Mixtures

In a series of experiments, 2.5 g crystalline β -lactoglobulin was incubated with 75 g lactose in 500 ml of a sterile phosphate buffer (0.01 M, pH 7) at 50° in a stoppered flask. After 24, 48, 72, 96 and 168 h 100 ml samples were taken, which were dialyzed extensively against distilled water in a cold room (4°); the clear brown solutions were dried by lyophilization.

The optical and analytical data of the various fractions have been collected in table IV.

The ultraviolet absorption spectra of the modified protein samples were found to be significantly altered as compared to the protein blanks submitted to the same treatment in the absence of sugar. The difference curves revealed prominent peaks at 250 and 305 nm. Similar peaks were also observed in the difference curves of the active fractions VM 5, 6 and 7 (fig. 2).

Moreover solutions of the hexulose-protein samples showed a characteristic fluorescence upon irradiation with ultraviolet light of 365 nm wavelength. From the data in table IV it may be observed that during the browning reaction free ϵ -amino groups are being blocked by condensation with sugar, as indicated by a fall in the amount of

Table IV. Analytical data and skin reactivities of modified β -lactoglobulin preparations

Preparation	Incubation time at 50° [h]	Absorption [E] % 305 nm	Fluorescence intensity ¹ [450 nm]	Available lysine content ² [%]	Hexose content ³ [%]	Number of peptide bonds split with trypsin, per mol. of protein ⁴	Skin reactivity ⁵ [units/mg]
crystalline β -lactoglobulin	0	0.60	0.6	12.0	0.1	17-18	<100
VM A	24	1.58	2.3	11.0	1.9	13	3,300
VM B	48	2.04	3.4	10.3	2.9	11	11,000
VM C	72	2.54	4.8	9.4	3.0	10	11,000
VM D	96	2.88	6.0	8.9	3.3	9	9,000
VM E	168	3.14	7.7	7.3	4.15	9	7,000
VM F (control)	168	1.44	2.3	11.3	0.1	17	<100

¹ Fluorescence intensity in arbitrary units (amplified 10 times) of 0.05% solutions in 0.01 M, pH 7 phosphate buffer.

² Assayed after CARPENTER [21].

³ Assayed after BRÜCKNER [15], expressed as galactose.

⁴ Number of peptide bonds split the first 60 min after the addition of trypsin to the reaction vessel; the mixtures were pre-incubated during 60 min at pH 8.5-9 to denature the protein.

⁵ Skin reactivities in units/mg dry weight material, average of four atopic patients with a positive skin test to milk (table I).

available lysine. With an increasing ultraviolet absorption at 305 nm and with an increasing fluorescence intensity the available lysine content was observed to decrease, while the number of peptide bonds split under the action of trypsin diminished significantly.

The modified β -lactoglobulin preparations obtained were assayed for skin reactivity in atopic individuals with positive skin reactions to milk. The average skin reactivities have been given in table IV. The data clearly showed that the specific skin reactivity of β -lactoglobulin is considerably increased by the incorporation of lactose, despite the marked alterations induced in the protein molecule [14, 19]. The amount of substance required for a positive intradermal reaction fell from 10 μ g for crystalline β -lactoglobulin to 0.10 μ g for the protein-lactose preparations VM B and C, whereas 10 μ g of the control protein heated without lactose (VM F) was found to elicit no positive reactions. It may be noticed from table IV that during longer heating periods the skin reactivity of the 1-deoxy-lactulose- β -lactoglobulin preparation obtained gradually diminished. In the preparations with maximum activity (VM B and C) about 4-6 lactulose groups have been incorporated in the β -lactoglobulin molecules, condensation of more ϵ -amino groups with sugar did not lead to an increased reactivity. The decrease of activity during longer heating periods can probably be attributed to the occurrence of marked alterations within the protein molecular structure, either by heat denaturation effects or by changes of the charge distribution. Furthermore degradation reactions may occur in the bound lactulose groups [5, 6, 19]. The most interesting point of our studies is that the selective coupling of lactose to the ϵ -amino groups of lysine side chains in the β -lactoglobulin molecules does increase the specific activity of the protein in skin tests in milk hypersensitive individuals.

In combination with the results of the fractionation studies, these findings suggest that the wheal and erythema phenomena observed after injection of the fractions VM 5, 6 and 7 are due to, or markedly influenced by, by browning reactions modified β -lactoglobulin molecules, the presence of which could be demonstrated in these active fractions.

Discussion

Recent studies on the chemical and spectral properties of purified inhalant allergens have suggested that these allergens are characterized

by the incorporation of N-substituted-1-amino-1-deoxy-2-ketose residues in the molecular structure [6, 8, 10]. Interestingly, the optical and chemical properties of the skin reactive fraction isolated from ripe tomato fruits were found to resemble closely those of purified inhalant allergens [15, 16]. In the case of the active tomato preparation evidence could be presented that the biological activity of the glycoprotein material is associated in some way with the N-glycosidically bound sugar residues. It was observed, for example, that by mild oxidation of the 1-amino-1-deoxy-2-ketose groups with $K_3Fe(CN)_6$ at pH 5.6, a product is obtained with a markedly decreased activity [16]. Circumstantial evidence indicates that also in inhalant allergens N-glycosidic protein-sugar bonds play a role in the elicitation of skin responses in atopic individuals [7].

The significance of the sites of N-glycosidic coupling for the biological activity of allergenic proteins became especially evident in the present study. In model studies, using β -lactoglobulin-sugar mixtures, we were able to show that the skin reactivity of bovine β -lactoglobulin in atopic persons with positive skin reactions to milk may be increased a hundredfold by reacting the protein with lactose. This result is even more striking because allergic reactions are thought to be mediated by specific reaginic antibodies, while the interaction with specific antibody in other immunological systems is usually diminished or lost after physical or chemical modification of the antigen.

Though it may be tempting to assume that these N-substituted-amino-deoxy-ketose groups account for the allergenic activity, or act as allergenic determinant sites, at the present stage only the conclusion that these groups in some way are involved in the sequence of reactions leading to the ultimate wheal and erythema phenomena observed in challenged skin sites of atopic individuals may be drawn. It is significant, in this respect, that BERRENS [11] observed that atopic allergens inhibit specifically the enzyme aminopeptidase-B and it was shown that the inhibitor activity correlates with the degree of browning of the allergens. Aminopeptidase-B is involved in the conversion of kallidin-10 into bradykinin (so-called kinins which are vaso-active and smooth muscle stimulating); they are released in inflammatory processes, for example after antigen-antibody reactions. Both kinins are usually rapidly inactivated by cleavage of the C-terminal arginine of bradykinin by the kininase carboxypeptidase; kallidin-10, however, is only inactivated after conversion into bradykinin. Should aminopeptidase-B be inhibited an overflow of kallidin-10 may occur, which

leads to an intensification of the primary response in skin sites challenged with allergen preparations.

Accordingly, incorporation of lactose groups in β -lactoglobulin may lead to an intensification of the wheal and erythema phenomena and therefore to increased skin reactivities. However, the probable intensifying effect of N-glycosidically bound deoxy-lactulose does not offer on altogether satisfying explanation that positive skin reactions to (modified) β -lactoglobulin occur. Besides the fact that several other protein substances incorporating N-glycosidically bound hexulose residues did not show comparable skin reactivity in atopic individuals, modified β -lactoglobulin did not show activity in other atopic and non-atopic persons either [17]. Current research indicated that glycoproteins need additional requirements for being able to elicit hypersensitivity reactions and clinical symptoms in atopic individuals.

Regarding the skin reactive principles which have been isolated thus far from foodstuffs especially some interesting facts arise by comparing the physicochemical properties of these substances. The most important data of the active fractions obtained from cod [2, 3], ripe tomato fruit [15, 16], egg white [18] and cow's milk have been collected in table V.

Interestingly, the active protein fractions were found to be stable to heat treatment and are not or only slightly modified during incubation at pH 2 and 37°. In addition, the preparations isolated from egg white and tomatoes proved to resist the action of trypsin and chymotrypsin. These findings indicate that the active protein components will have a relatively great chance to reach (via the oral route) the blood stream and the reticulo-endothelial system unaltered, even after heat-processing of the foods in question.

Furthermore it may be observed from table V that the molecular weights of the active components are of the same order of magnitude, ranging from 18,000 for the major allergen fraction from cod to 36,000 for the β -lactoglobulin fraction of cow's milk. This finding substantiates the earlier observation of STANWORTH [49] that the sedimentation coefficients of inhalant allergens fall in the narrow range of $S_{20} = 2.2$ –3.4. It is likely therefore, that allergenic activity is narrowly restricted to (glyco)proteins with a molecular weight of 20,000–40,000, implicating a discrimination between most normal protein antigens and atopic allergens leading to reagin production and symptoms in atopic individuals. In this respect the major allergen fraction from milk has similar characteristics as the active components isolated from materials of widely different origin.

Table V. Properties of skin reactive components of some foods

Food	Active component	mg of isolated component per 100 g	Skin reactivity: minimum amount of material to elicit a positive skin reaction	Mol. weight	Stability during heating at pH2 at 100°	Resistance against the action of chymotrypsin	Remarks
Fish, cod [2, 3]	sarcoplasma proteins	200	0.0001 μ g	18,000	+	?	The major components (Hanoir proteins) are not present in the muscle tissue of mammals.
Tomatoes [15, 16]	glycoprotein fraction	2.5	0.15	20,000–30,000	+	+	Produced during the storage and the ripening of the fruits.
Egg (white) [18]	ovomucoid fraction	225	0.0025	31,500	+	++	Ovomucoid inhibits the proteolytic activity of trypsin; egg white also contains a chymotrypsin inhibitor.
Cow's milk past.	β -lactoglobulin modified	100	0.10	36,000	+	+ ¹	Not present in breast milk.

¹ Native β -lactoglobulin is only slightly susceptible to enzymes.

Studies performed by GOLDMAN [25, 26] and PARISH [41] presented additional evidence that β -lactoglobulin indeed may be considered as the most active fraction of cow's milk. As described in the introduction of this paper GOLDMAN [26] did skin reactions in atopic and non-atopic children with casein, bovine serum albumin, α -lactalbumin and β -lactoglobulin. Only in nine atopic children were strong positive skin reactions observed to one or more antigens (of a total of 89 atopic individuals). These patients were found clinically hypersensitive to milk by means of oral challenges with milk and milk fractions. From the reported data the average skin reactivities may be calculated for the four protein preparations. In the group of the aforementioned 9 patients the average activity of β -lactoglobulin was found to be 10–100 times higher than those of the other proteins. In contrast the average skin reactivities found in the other groups of patients proved to be equal for all four preparations.

Recently PARISH [41] studied the release of histamine and slow-reacting-substance after challenge of human lung tissue sensitized passively with reagin *in vitro*; sera used to sensitize the lung fragments were from two patients allergic to milk who gave an immediate skin response to milk protein. He stated that the sera contained reagin to β -lactoglobulin as detected by passive cutaneous anaphylaxis in baboons, no reactions were found to casein, bovine serum albumin and α -lactalbumin. None of the sera sensitized guinea-pig skin by passive cutaneous anaphylaxis, indicating that in the sera little IgG antibody to milk proteins was present. Both histamine and slow-reacting-substance were being released by challenges with β -lactoglobulin, but not by the other three milk protein fractions.

So far, the evidence is in favour of the assumption that the main allergenic activity of pasteurized milk is associated with the β -lactoglobulin fraction. Our results indicate moreover, that the biological activity of β -lactoglobulin may be considerably increased by the incorporation of lactulose residues within the protein molecules.

Since browning reactions between lactose and proteins have been shown to occur during pasteurization, storage and household handling [34, 42] this finding also implicates that these treatments may lead to an increase of the allergenic activity of cow's milk. Therefore care should be taken in using heated milk preparations as hypo-allergenic foodstuffs for atopic individuals.

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Summary

In this paper a description is given on the identification of the skin reactive component in pasteurized and raw cow's milk.

Isolation studies showed the major allergenic activity to reside largely in the β -lactoglobulin fraction (molecular weight 36,000, hexose content 0.1–1.1%). However, crystalline β -lactoglobulin was found to exert much lower activity in atopic individuals with positive skin reactions to cow's milk. Furthermore chemical and spectroscopic analysis indicated the presence of traces of 1-deoxy-hexulose-proteins in the crude β -lactoglobulin fractions. Consequently an attempt was made to increase the specific activity of crystalline β -lactoglobulin by synthetically incorporating N-glycosidically bound sugar into the protein molecule. During these model studies it was found that the specific activity of β -lactoglobulin is considerably increased by the browning reaction with lactose.

The results are discussed in relation to the findings of other authors, especially with regard to the chemical characteristics of inhalant allergens and the skin reactive fractions isolated from foods.

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