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Interaction between κ-Casein and β-Lactoglobulin: Possible Mechanism

Zahurul Haque, Magnus M. Kristjansson, and John E. Kinsella*

κ-Casein (κ-C) and β-lactoglobulin (β-Lg) interact to form a κ-C/β-Lg complex (A_4) apparently composed of three molecules of β-Lg and one molecule of κ-C. In a freshly dispersed equimolar mixture of native κ-C and β-Lg maintained at 25 °C in 20 mM imidazole at pH 6.8, approximately 14% occurred as a κ-C/β-Lg A_4 complex which increased to about 40% when 20 mM ethylene glycol bis(2-aminoethoxy)-*N,N,N',N'*-tetraacetic acid (EGTA) was added. The EGTA apparently caused a structural change that resulted in a β-Lg complex (A_3) that was highly reactive with monomeric κ-C. This reactivity was quenched in the presence of 2.3 M urea. With time, this A_4 complex became stabilized by covalent bonds. Heating at 70 °C increased the rate of formation of the covalently bonded κ-C/β-Lg complex. A possible mechanism for the interaction of κ-C with β-Lg involves the formation of a β-Lg homotrimer (A_3) complex via hydrophobic interactions that interacts with a κ-C molecule.

Nonfat dry milk (NDM) is a significant source of functional protein for the food industry (Kinsella, 1985). The cheese industry represents a large potential market for NDM, if the NDM could be used for the manufacture of rennet curds and cheeses. However, the heat treatment used in the manufacture of NDM reduces the susceptibility of the κ-casein to the normal action of chymosin because of complex formation between κ-C/β-Lg (Sawyer, 1968; McKenzie, 1971). This is because in the routine manufacture of NDM milk rceives a prior heat treatment to cause interaction of κ-C with β-Lg and eliminate the loaf-depressing effect in NDM intended for use in the baking industry (Guy, 1970). Unfortunately, the same heating conditions may be used for the preparation of NDM for other than baking applications, thereby limiting its potential use in rennetted cheese/curd type products.

Though many of the heat-induced interactions occurring in milk have been elucidated, not all the interactions occurring within and between protein components are understood. Heat treatment may cause denaturation particularly of whey proteins, resulting in adsorption to caseins and to possible interaction between β-lactoglobulin (β-Lg) and κ-casein (κ-C) via hydrophobic interactions (Doi et al., 1983), disulfide/sulfhydryl interchange reaction, and/or via thiol oxidation (Purkayastha et al., 1967), resulting in

Institute of Food Science, Stocking Hall, Cornell University, Ithaca, New York 14853.

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cross-linking between β-Lg and κ-C on the micellar surface (Kalab et al., 1983; Heertje et al., 1985). The oxidation of thiol groups, particularly of β-Lg, may be one reason for the elimination of the loaf-depressing factor in milk powders. However, the complex formation between κ-C and β-Lg reduces the susceptibility of the milk protein to rennin action (Tobias et al., 1952; Slatter and van Winkle, 1952; McGugan et al., 1954; El-Negoumy, 1974).

Despite the detailed research, the mechanism of the interaction is not clear. Because of the desirability of controlling and/or minimizing the interaction between κ-C and β-Lg during the manufacture of NDM, more detailed information about the nature and the sequence of the reaction is needed in order to minimize or control it. This paper looks into the association/dissociation pattern of the native and heated κ-C and β-Lg, describes the isolation of the complexes by high-performance liquid chromatography (HPLC), and proposes a mechanism for the complex formation between κ-C and β-Lg. Size-exclusion chromatography has been chosen as the principal tool because it has been used traditionally for analyzing proteins (Gruber et al., 1979; Ui, 1979). Since its introduction, chemically bonded controlled-porosity glass bead columns have been used in HPLC for separating proteins on the basis of molecular mass (Fischer et al., 1978).

EXPERIMENTAL SECTION

Materials. Fresh skim milk (Holstein) was obtained from the Cornell Dairy Plant. Ethylene glycol bis(2-aminoethoxy)-*N,N,N',N'*-tetraacetic acid (EGTA) and 2-mercaptoethanol (2-ME) were from Sigma Chemical Co., St. Louis, MO. Recrystallized urea was from Pierce Chemical Co. Filter type HA of size 0.45 μM was from Millipore Corp., Bedford, MA. Molecular weight calibration proteins were obtained from Pharmacia, Uppasala, Sweden. Ultrafiltration membrane (PM-10) was from Amicon, Lexington, MA. Bio-Rad protein assay kit was from Bio-Rad Laboratories, Richmond, CA. All other chemicals were of analytical grade.

Methods. *Protein Purification and Thermal Treatment.* κ-C and β-Lg were purified from delipidated skimmed milk as described. β-Lg was crystallized by the method of Aschaffenburg and Drewry (1957), and κ-C was obtained as described by Zittle and Custer (1964). Protein concentration was determined from the relationships $E_{280}^{1\%,1cm}$ = 12.2 and 9.6 for κ-C and β-Lg, respectively (Zittle and Custer, 1964; Townend et al., 1964).

Buffers. Imidazole buffer (20 mM) at pH 6.8 was used without (buffer A) or with the following additives: 20 mM EGTA (buffer B); 10 mM 2-Me (buffer C); 5% ethylene glycol and 0.5 M NaCl plus 10 mm 2-Me (buffer D); 2.3 M urea and 20 mM EGTA (buffer E); 2.3 M urea and 20 mM EGTA plus 10 mM 2-ME (buffer F). The same buffers were used for sample preparation and HPLC elution. Urea was never used during the heating experiments. The standard HPLC buffer unless otherwise specified was buffer E [imidazole buffer, pH 6.8 (buffer A) with 2.3 M recrystallized urea and 20 mM EGTA]. Calcium was not added to any of the above buffers.

Sample Preparation and Separation of Native Proteins. To observe the association/dissociation behavior of the unheated native proteins, κ-C and β-Lg were dissolved separatly (1 mM) in one of the above-mentioned buffers, equilibrated to 25 °C for 30 min, and analyzed by high-performance liquid chromatography (HPLC). To study the κ-C/β-Lg mixture the individual protein solutions were mixed in a 1:1 ratio to give a total protein concentration of 1 mM and analyzed immediately by HPLC. Aliquots of the individual protein solution or the mixture were diluted fourfold, 2.5 μL [i.e. 11.5 μg of protein (assuming a mean molecular weight of 18 000)] was injected into the HPLC using a precision syringe, and the proteins were eluted with the reaction buffer, i.e. without urea. HPLC analyses were conducted at 25 °C.

Heat Treatment and Separation of Heated Samples. In the heating experiments, the freshly mixed κ-C/β-Lg mixture was heated at 70 °C in thin-walled glass vials (Haque and Kinsella, 1987a) for specific periods, and the reaction was stopped by chilling to 4 °C by fourfold dilution with reaction buffer at 4 °C. The samples were analyzed by HPLC as described above.

HPLC Separation and Fractionation. The molecular interaction and the apparent molecular mass of the homogeneous (κ-C/κ-C and β-Lg/β-Lg) and heterogeneous (κ-C/β-Lg) complexes were determined by HPLC using a Waters M-45 solvent delivery system connected to a variable-wavelength detector through a glass bead hydrophilic gel filtration column (TSK gel G3000SW) (Toyo Soda, Tokyo, Japan), a 60 cm × 7.5 mm (i.d.) column for molecular weight determination, and a 30 cm × 7.5 mm (i.d.) column for detecting molecular aggregation. The flow rate was 1 mL/min at 25 °C, and detection was at 280 nm.

The areas of the separated peaks were integrated with a Hewlett-Packard 3390A integrator, and the eluted protein fractions were pooled and concentrated with an Amicon ultrafiltration cell fitted with a PM-10 (10 kDa cut) membrane. The concentration of the pooled fractions was determined by the Bio-Rad Protein Assay. The molecular masses of the various fractions were determined by comparing their retention times with those of standard proteins.

Polyacrylamide Gel Electrophoresis (PAGE). In order to determine the composition of the proteins in the peaks eluted from HPLC, SDS-PAGE was carried out in 7.5% gels in the presence of 10 mM DTT. The protein samples were heated at 90 °C for 3 min in 2% (w/v) SDS and 50 mM 2-ME to bring about complete dissociation prior to the SDS-PAGE. About 30 μg of the pooled protein fraction was applied to each tube well. The current was controlled at 5 mA/gel tube for about 1 h at 20 °C. Staining/fixing was done with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 25% TCA for 30 min, and destaining was achieved with methanol–acetic acid–water (20:7:73, v/v/v). The κ-C/β-Lg mixture was pretreated for 30 min in 10 mM 2-ME and then separated in SDS-PAGE containing 1 mM DTT. Following staining and destaining, the gels were stored in 5% acetic acid and scanned on a densitometer (540 nm) (E.C. Apparatus Corp.) fitted to a HP 3392A integrator.

RESULTS AND DISCUSSION

Profile of Typical HPLC-Separated Peaks. A typical HPLC profile of an unheated κ-C/β-Lg mixture eluted using imidazole buffer (buffer E) at 25 °C revealed three peaks (Figure 1) eluting at 6.7 (A_4), 11.4 (A_3), and 15.6 (A_2) min, respectively. A fourth peak (A_1) has not been shown here and appeared after a retention time of 20 min only in the presence of 10 mM 2-ME as the slowest eluting fraction. Heated κ-C/β-Lg mixtures gave similar retention times for all the fractions; only the relative peak sizes changed.

Standard reference proteins were chromatographed in the standard HPLC buffer containing 2.3 M urea and 20 mM EGTA (buffer E) and a plot of the log molecular weight vs. retention time (Figure 2) was used to determine the degree of polymerization (apparent molecular mass) of the proteins. For these experiments a 60 cm × 7.5 mm column was used compared to the 30 cm × 7.5 mm column

Table I. Percentile Distribution of Various Complexes of Unheated κ-Casein, β-Lactoglobulin, and κ-Casein/β-Lactoglobulin Mixture following Interaction under Different Buffer Conditions[a]

HPLC buffers	β-Lg				κ-C				κ-C/β-Lg			
	A1	A2	A3	A4	A1	A2	A3	A4	A1	A2	A3	A4
(A) imidazole,[b] pH 6.8, 20 mM	0	99	T	0	11	53	T	32	0	55	30	14
(B) A[b] + EGTA (20 mM)	0	50	50	0	0	90	0	10	0	60	0	40
(C) A[b] + 2-ME (10 mM)	18	80	0	T	67	22	0	11	41	20	18	20
(D) C[b] + 0.5 M NaCl + ethylene glycol (5%)	97	T	0	T	96	T	0	0	92	5	T	10
(E) C[c] + EGTA (20 mM) + urea (2.3 M)	10	90	0	0	0	100	0	0	0	85	10	4
(D) E[c] + 2-ME (10 mM)	22	75	0	0	72	26	0	0	68	25	6	0

[a] Numbers indicate normalized percentages of detected protein. κ-C, β-Lg, and κ-C/β-Lg represent κ-casein, β-lactoglobulin, and the κ-casein/β-lactoglobulin mixture. T represents trace amounts. [b] Sample and HPLC buffer. [c] Sample buffer was buffer B.

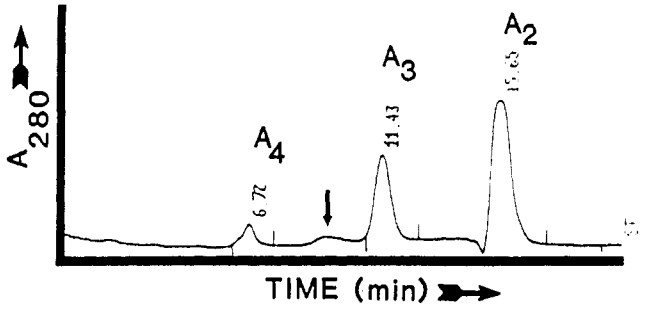

Figure 1. Typical HPLC separation profile of an unheated κ-C/β-Lg mixture using the standard HPLC buffer containing 20 mM imidazole, 2.3 M urea, and 20 mM EGTA on a 30 cm × 7.5 mm (i.d.) G3000SW column (Toyo Soda). The first, second, and third peaks correspond to A_4, the κ-C/β-Lg tetrameric complex; A_3 corresponds to the β-Lg trimeric complex; and the A_2 complex corresponds to (κ-C and β-Lg). The arrow indicates the retention time (9.5 compared to 11.4 min) of the β-Lg A_3 complex in the absence of EGTA in the buffer.

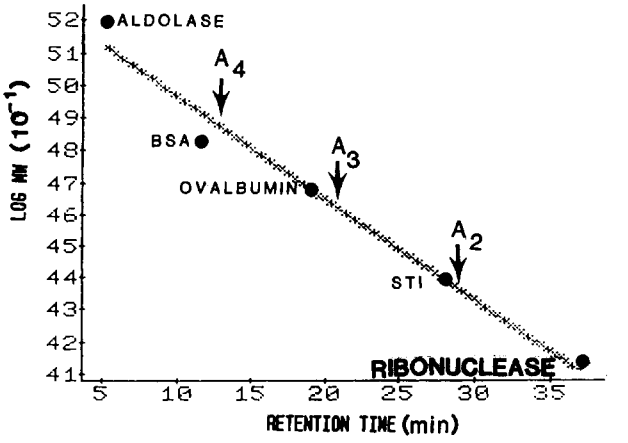

Figure 2. Standard HPLC curve using protein standards of known molecular weights. A longer column (60 cm × 7.5 mm) was used for these standard runs (see Methods). Aldolase (158 000), bovine serum albumin (BSA) (67 000), ovalbumin (45 000), soy trypsin inhibitor (STI) (22 000), and ribonuclease (13 000) have been used. A_4, A_3, A_2, and A_1 represent the first, second, third, and fourth peaks (composition same as in Figure 1). Buffer was the same as in Figure 1.

used for the rest of the study (see Methods). The elution positions of A_4, A_3, A_2, and A_1 indicated that they represent a tetramer, trimer, dimer, and monomer of either κ-C or β-Lg assuming a common molecular weight of 18 500. It should be emphasized that the elusion position may be influenced by the changes in the hydrodynamic radius of the molecules especially when elusion conditions are changed (Haque et al., 1987). However, the marked difference in the elusion positions of A_2, A_3, and A_4 and appearance also of the A_1 on addition of 10 mM 2-ME indicated that these fractions represented different states of molecular association.

To determine the composition of the separated peaks, SDS-PAGE of the proteins in each of the pooled isolated peaks was carried out for the κ-C/β-Lg mixture. The A_4 was composed of κ-C and β-Lg; A_3 was composed solely of β-Lg, and A_2 was composed of κ-C and β-Lg homodimers (Figure 3). From the SDS-PAGE experiments in the presence of 2-ME, the A_4 complex was composed of κ-C and β-Lg in the ratio 1:3. The stoichiometry of the interaction was based on the HPLC elution positions of the various fractions and on the degree of staining of the gel electrophoretic bands representing the components of these fractions. β-Lg trimers have previously been detected in red deer milk (McDougall and Stewart, 1977). Long et al. (1963) reported a κ-C to β-Lg ratio (1:2:2) in a complex formed after heating the protein mixture for 20 min at 85 °C.

Association/Dissociation Behavior of Unheated Proteins. The association/dissociation behavior of unheated κ-C, β-Lg, and K-C/β-Lg mixtures at pH 6.8 were studied by HPLC at 25 °C in various buffers (Table I). β-Lg existed only in the dimeric state whereas κ-C was distributed in the order A_2, A_4, and A_1 states when both reaction and HPLC buffers were 20 mM imidazole, pH 6.8. However, in equimolar κ-C/β-Lg mixtures, the proteins

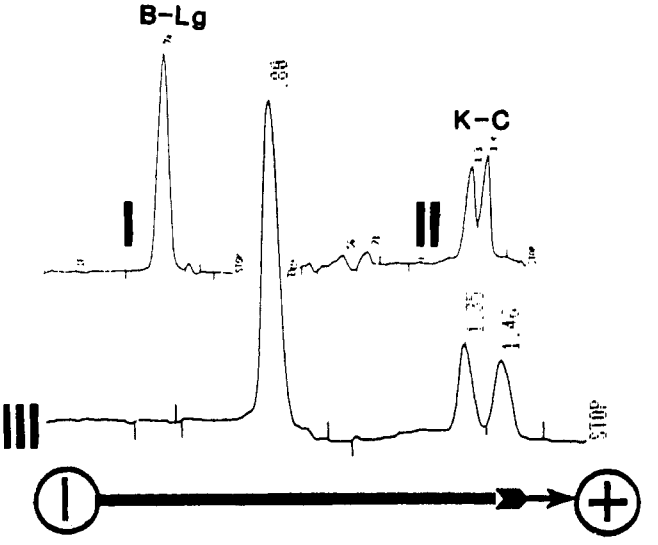

Figure 3. Densitometric scans of SDS-PAGE gels. I–III correspond to pooled A_3 (β-Lg was the same), κ-C alone, and pooled A_4, respectively.

existed as A_2 and A_4 (85:14) forms and the A_3 occurred in trace amounts. Upon addition of EGTA to both the reaction and HPLC buffer, in the absence of κ-C, β-Lg existed as an equilibrium between A_2 and A_3 complexes. In the presence of κ-C the A_3 complex disappeared completely with a concomitant increase in the A_4 complex. κ-C never existed in the A_3 state. Pretreatment of the individual protein solution for 20 min with 10 mM 2-ME caused the β-Lg to remain predominantly in the A_2 state while κ-C

still existed in the A_4 and A_2 states. When ethylene glycol (5%, v/v), 10 mM 2-ME, and 0.5 M NaCl were added to this buffer containing the proteins (either combined or separated) the proteins occurred predominantly in the A_1 form, i.e. in the monomeric form.

When the HPLC standard buffer containing 2.3 M urea and 20 mM EGTA (buffer E) was used as the HPLC separation buffer, the individual proteins were dissociated by urea and eluted in their dimeric state (Table I). The A_4 and A_3 complexes were detected only in the κ-C/β-Lg mixture. The urea-resistant κ-C/β-Lg A_4 complex was not dissociated by Triton X-100 (0.1%, v/v) nor by ethylene glycol (5%, v/v) in the standard HPLC buffer containing 2.3 M urea (2.3 M), but it was dissociated upon addition of 10 mM 2-ME to the sample 5 min prior to injection into the HPLC (buffer F). The urea-resistant A_4 complex was evidently stabilized by disulfide bonds that were reduced by the thiol reagent. However, after 24 h of storage, the S–S bonds were less susceptible and were reduced only after treatment with 2-ME for >30 min, reflecting some conformational changes protecting the disulfide bonds.

The SDS-PAGE of the fractions separated from the κ-C/β-Lg mixture showed that A_3 appears to be composed of β-Lg only (Figure 3-I), and the HPLC retention time of A_3 was significantly increased by EGTA, indicating some change in the molecular structure affecting the formation of the κ-C/β-Lg A_4 complex (Table I). However, the decrease in retention time of A_3 in the absence of EGTA was significant but not large enough to indicate further association of another monomer of β-Lg.

The EGTA-altered β-Lg was more reactive with the κ-C molecule to give the A_4 complex. This occurred even at 25 °C, and the reaction was quenched by urea (buffer E). On the basis of previous studies, there is no structural alteration of the β-Lg molecule at the level of urea used (2.3 M) (Cupo and Pace, 1983). Hence, the urea in the HPLC buffer apparently disrupted noncovalent intermolecular associations in the κ-C/β-Lg A_4 complex.

Since EGTA is a specific chelator of divalent cations and Ca^{2+} is the predominant divalent cation in milk, an explanation of the increased reactivity of the EGTA-altered A_3 may be related to Ca^{2+}–induced modulation of the conformation of β-Lg. It is conceivable that trace amounts of the predominant divalent cation in milk, Ca, remained bound to the proteins even after the purification steps and caused conformational changes of the β-Lg molecule. Addition of Ca^{2+} to the reaction mixture significantly decreased the reactivity of the A_3 with κ-C, and the reactivity was totally suppressed when the mole ratio of Ca^{2+} to protein reached 0.2 (Haque and Kinsella, 1987b). Aggregation of β-Lg is extremely sensitive to the presence of Ca^{2+} (Darling, 1980). Doi et al. (1981) also reported that calcium hinders the complex formation between κ-C and β-Lg.

Calcium affects the heat-induced insolubilization of whey proteins (de Rham and Chanton, 1984) and among the whey proteins, β-Lg is the most sensitive to Ca^{2+} (Varunsatian et al., 1983). The amount of Ca^{2+} required to induce aggregation of β-Lg was equivalent to the net charge of β-Lg (DeWit, 1981). At high-pH or low-Ca^{2+} concentration, the aggregation of β-Lg is retarded and the interaction with κ-C was deemed to be more faborable (Darling, 1980).

Effect of Heat Treatment on Formation of the κ-C/β-Lg Complex. Heating of κ-C/β-Lg mixture and subsequent HPLC separation in 20 mM imidazole buffer without EGTA showed an initial decrease in the A_2 peak with a concomitant increase in A_3. The A_1 protein was detected briefly (Figure 4A), indicating heat-induced dissociation. It is possible that the β-Lg component of the predominantly dimeric protein (A_2) mixture dissociated to the monomeric state, which then gave the A_3 complex.

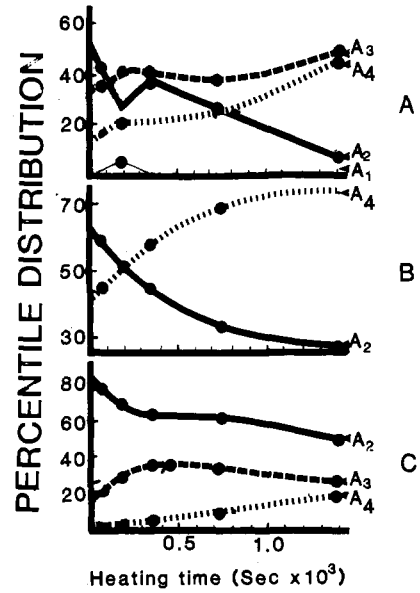

Figure 4. Percentile distribution of the different aggregates formed following heating of the κ-C/β-Lg mixture: (A) 20 mM imidazole buffer, pH 6.8; (B) (A) plus 20 mM EGTA; (C) (B) plus 2.3 M recrystallized urea (standard HPLC buffer).

Georges et al. (1962) demonstrated by light scatter measurements that β-Lg shows increasing dissociation to the monomer at a given pH in the region pH 6–9 as the temperature is increased from 20 to 45 °C, and above 55 °C the oligomers usually occurring at neutral pH are almost completely dissociated (Dupont, 1965). A similar observation was made by DeWit and Swinkels (1980); they reported heat-induced denaturation of β-Lg is first order (65–72 °C) and the initial lag of several minutes was attributed to a preceding monomolecular transition of β-Lg from a reversible altered conformational state to an irreversible disordered state (Pantaloni, 1964).

The A_3 complex became predominant following 90 s of heating. The concentration of A_4 species remained essentially the same for about 720 s after which there was a gradual increase up to 1440 s (24 min) when it equaled the A_3 complex. When EGTA was added to both the reaction and HPLC buffer, the initial A_2 to A_4 ratio (3:2) changed rapidly and the ratios were 1:1 and 1:3 at 180 and by 1440 s, respectively (Figure 4B), reflecting increased aggregation.

Dziuba (1979) reported that heating first causes β-Lg aggregation, and the β-Lg aggregate then reacts with κ-C. Heat-induced β-Lg complex is insoluble but becomes soluble when associated with κ-C (Doi et al., 1981). Electron micrographs show that heated β-Lg polymerizes and forms a network structure but upon adding κ-C, however, the complex size became small (Heertje, 1986).

The κ-C/β-Lg interaction was enhanced by increasing the temperature. However, heating did not significantly alter the HPLC retention time of the A_3 complex, indicating little change in structure, and the enhanced reaction rate at 70 °C apparently reflected increased potential for hydrophobic interactions at that temperature (Scheraga et al., 1962).

The difference between the unheated and heated A_3 complex was the apparent irreversibility of the transition from the dimeric to the trimeric state (β-Lg) in the case of the heated A_3 complex. Whereas the unheated A_3 was

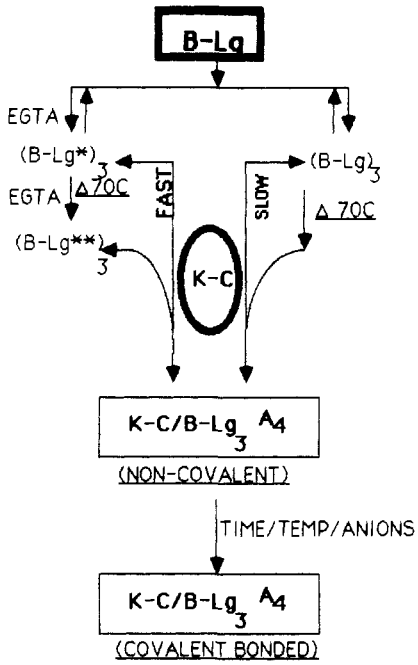

Figure 5. Effect of heat and buffer components on the formation of the β-Lg A_3 complex and its subsequent interaction with the κ-C molecule. (β-Lg)* denotes the conformational state in the presence of EGTA that was reactive with κ-C, and (β-Lg)** denotes the same species "activated" by heat treatment.

easily dissociated (Table I), the heated A_3 complex was dissociated during SDS-PAGE following pretreatment at 90 °C for 3 min in a buffer containing 2% SDS (w/v) and 50 mM 2-ME. This indicated a very tenacious noncovalent and/or covalent association.

Formation of Urea-Resistant A_4 Complex. Since urea disrupted the noncovalent interaction of the β-Lg A_3 with a κ-C, the standard HPLC buffer (i.e., standard reaction buffer plus 2.3 M urea) was used to detect the formation of covalently stabilized κ-C/β-Lg A_4 complex (Figure 4C). The A_2 complex decreased upon heating with a concomitant increase in A_3 until at about 180 s of thermal treatment a critical concentration of the A_3 was reached (38%). The urea-resistant A_4 complex gradually increased with a corresponding decrease in A_3 and A_2. The rate of formation of urea-resistant A_4 complex was slow and linear with time.

The κ-C/β-Lg interaction occurred even when the experiments were carried out under nitrogen. It is conceivable that a reactive -SH group in the hydrophobic cleft of the β-Lg molecule (Sawyer et al., 1985) became available after heat alteration of the β-Lg molecule and this then engaged in thiol–disulfide interchange as observed by others (Larson and Jenness, 1952); Stauff and Uhlein, 1958). The data indicate that κ-C molecule initially interacted with the β-Lg A_3 complex hydrophobically to give the urea-sensitive κ-C/β-Lg A_4 complex, enhancing the subsequent chance for covalent interaction.

Proposed Mechanism. A scheme outlining formation of A_4 complex under the experimental conditions is shown in Figure 5. The first stage in the thermal interaction involved the formation of the urea-stable A_3 (β-Lg_3) complex that was less reactive with κ-C in the presence of Ca^{2+}. In the presence of EGTA, a more reactive A_3 complex interacted with one molecule of κ-C to give the A_4 complex (Figure 5). The size/shape of the A_3 complex affects the rate constant of A_4 formation (Haque et al., 1987). Heating at 70 °C increased the rate of A_4 formation by increasing the potential for hydrophobic interaction. The A_4 complex formed initially was dissociated by 2.3 M urea to A_3 and κ-C, but with time the complex became stabilized by covalent bonds to give the urea-resistant A_4 complex that was cleaved only when 2-ME was added to the urea-containing buffer. This covalently bonded A_4 complex was formed even when the system was depleted of oxygen by nitrogen flushing, suggesting that -SH/S-S interchange was involved.

κ-C is amphipathic and self-associates via hydrophobic interactions. The κ-C in solution (native) is in a micellar state (m = 30) under conditions used in these experiments (Vreeman et al., 1981). However, the basic unit of κ-C separable by the standard HPLC buffer (urea and EGTA) was a dimer (A_2), indicating that the dimer is the building block in the formation of the micelles. At 70 °C the concentration of the reactive κ-C monomer limited its availability for interaction with the A_3, and this may counteract the driving force for the interaction. The state of self-association of κ-C may therefore play an important role in the interaction. Alterations in pH, concentration, ionic, or temperature conditions could bring about changes in the state of self-association of κ-C. A case in point is the holding temperature of heated milk and its effect on subsequent rennetability, i.e. the lower the temperature of holding, the greater the decrease in rennetability (Powell, 1936). In other words, κ-C/β-Lg interactions in heated milk are greatest at lower holding temperatures at which the tendency for self-association of κ-C is less and hence more monomeric κ-C may be available to react with β-Lg. We observed a similar trend with a heated κ-C/β-Lg mixture (Haque et al., 1987). It should however be emphasized that the stoichiometry of the reaction is based on the HPLC elution profile of the various fractions and the staining intensity of the components of the different bands following SDS-PAGE of these fractions. Light scatter or analytical ultracentrifugation may be required to further establish this point.

Efforts to diminish interactions between κ-C and β-Lg should focus on the proposed two stages of interaction. A measure should be taken to prevent the formation of the more reactive form of β-Lg. However, such a measure would result in the precipitation of β-Lg/A_3 complex due to homopolymerization unless some amphipathic molecule is used to preferentially bind to and solubilize the A_3 complex. A second measure would be to induce polymerization of the κ-C molecule, thereby making the κ-C monomer less available for interaction with β-Lg A_3.

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Registry No. EGTA, 67-42-5; NH_2CONH_2, 57-13-6; imidazole, 288-32-4.

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A Continuous Spectrophotometric Screening Assay for Glucoamylase

Robert D. Sabin and Bruce P. Wasserman*

A continuous glucose oxidase–peroxidase based spectrophotometric assay suitable for the rapid screening of glucoamylase activity is described. The assay utilizes maltotetrose and the chromogens 3-(dimethylamino)benzoic acid (DMAB) and 3-methyl-2(3*H*)-benzothiazolinone hydrazone (MBTH). The assay was sensitive to glucoamylase levels of 0.05 μg/mL and was linear with respect to enzyme concentration to approximately 0.5 μg/mL.

Glucoamylase is a widely utilized exoenzyme that removes glucose units consecutively from the nonreducing ends of starch and oligosaccharides (Pazur and Ando, 1960; Reilly, 1979). Glucoamylase activity is defined as the quantity of glucose liberated from substrate per unit time. Methods commonly used for glucoamylase detection include reducing sugar determination (Somogyi, 1952; Bernfeld, 1955) and the enzymatic analysis of the glucose released by either the glucose oxidase–peroxidase method (Hugget and Nixon, 1957; Dahlqvist, 1961; Lloyd and Whelan, 1969) or the hexokinase–glucose-6-phosphate dehydrogenase (HK/GDH) coupled assay (Bergmeyer et al., 1974). Both analytical approaches have weaknesses. Reducing sugar methods are not valid indicators of glucoamylase levels when α-amylases or pullulanases are present. The glucose oxidase–peroxidase method is most often utilized as a fixed-point assay and frequently employs *o*-dianisidine, a carcinogen, as the chromogen. The HK/GDH method, also a fixed-point assay, can only be conducted at pH 7.0 or above. Since glucoamylase assays are conducted at pH 4.5, an adjustment of pH is required, thus necessitating a multistep assay. A continuous glucoamylase assay utilizing glucose oxidase–peroxidase with amylose as substrate has been reported (Pazur et al., 1971); however, this assay also employed *o*-dianisidine.

The development of a rapid and continuous spectrophotometric screening assay for detection of glucoamylase activity utilizing noncarcinogenic color reagents is highly desirable. Ngo and Lenhoff (1980) reported the use of 3-(dimethylamino)benzoic acid (DMAB) and 3-methyl-2-(3*H*)-benzothiazolinone hydrazone (MBTH) for measurement of peroxidase and peroxidase-coupled reactions. For glucose measurement, glucose oxidase is added to convert glucose and O_2 to gluconic acid and H_2O_2. Then, H_2O_2, MBTH, and DMAB reacted in the presence of peroxidase to form an indamine dye with an absorption peak of 590 nm. By either fixed-point or rate methods, this assay provided a sensitive measurement of glucose. The objective of this study was to adapt this reaction for a quick screening assay of glucoamylase-catalyzed glucose release from an oligosaccharide substrate. Since amylose is a substrate for interfering endo- and debranching α-amylases, its use was avoided and the purified maltooligosaccharide substrate maltotetrose was utilized instead.

MATERIALS AND METHODS

Materials. Purified *Rhizopus niveus* glucoamylases were obtained from Seikagaku Biochemicals (U.S. Distributor; Miles Biochemical Co., Elkhart, IN). DMAB and MBTH were purchased from Aldrich Chemical Co. (Milwaukee, WI). Maltotetrose, maltotriose, and maltose

Department of Food Science, New Jersey Agricultural Experiment Station, Cook College, Rutgers University, New Brunswick, New Jersey 08903.

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