Major Proteins of Beer and Their Precursors in Barley: Electrophoretic and Immunological Studies

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SDS-PAGE analysis revealed that what was considered the major protein of beer is actually formed by two polypeptides with the same molecular mass (~40 kDa), but different hydrodynamic volumes in their incompletely unfolded conformation. The two polypeptides share common properties, although the one with the more open conformation is associated with sugars, whereas the other is not. Immunoblotting experiments with polyclonal antibodies raised against the two electrophoretically purified polypeptides indicated that they are immunologically related and allowed the identification of their precursors in barley grain. These latter are two heat-resistant albumins whose electrophoretic behavior corresponded to that of beer proteins. These two albumins coincide with protein Z, the first member of the serpin superfamily described in plants.

Keywords: *Beer proteins; barley protein Z; electrophoresis; antibodies; glycated proteins.*

INTRODUCTION

Beer contains ~500 mg/L of proteinaceous material (Hejgaard and Kaersgaard, 1983), including a variety of polypeptides with molecular masses ranging from <5 to >100 kDa (Sørensen and Ottesen, 1978). These polypeptides, which mainly originate from barley proteins, are the product of the proteolytic and chemical modifications that occur during brewing. The importance of this kind of material in determining the quality characteristics of the finished product has been recognized for a long time. In particular, the polypeptides are thought to be involved in the mechanisms of both haze formation and foam stabilization. The latter is dependent, *inter alia*, on the molecular characteristics of the protein fractions. Of particular importance is the simultaneous presence of both hydrophilic and hydrophobic groups in the same molecular structure, which allows the molecule to enter the liquid layer between the bubbles and interact with both the gas and the hydrophobic groups of other molecules within the bubble wall. This behavior is typical of sugar-associated polypeptides, which seem to act as particularly strong foam stabilizers (Bamforth, 1985). In this context, the molecular flexibility is also considered an important factor (Le Meste et al., 1990) because the exposure of hydrophobic residues following the unfolding at the air–water interface improves foam formation and stability.

Among the proteinaceous material present in beer, the only component that has been well characterized as a relatively unmodified protein is a polypeptide with a molecular mass of ~40 kDa, called antigen 1. Antigen 1 is present in varying amounts (from ~20–170 mg/L) and is involved, at least in part, in beer foam and haze formation. This protein is comprised of two genetically different, but immunologically related polypeptides with the same electrophoretic mobility, called antigen 1a and 1b (Hejgaard and Kaersgaard, 1983). These antigens are derived from two different albumins present in barley grain, named proteins Z_4 and Z_7, which are coded by genes on chromosomes 4 and 7, respectively (Hejgaard, 1984). Proteins Z_4 and Z_7 have similar properties, although protein Z_7 has been identified with certainty only by immunological means. Protein Z_7 probably corresponds to the 39 kDa protein characterized by Lundgar and Svensson (1989). Protein Z_4 has been well characterized (Hejgaard and Kaersgaard, 1983), and its primary structure has been elucidated (Brant et al., 1990). More recently, another protein Z-related gene coding for protein Z_X has been described in barley (Rasmussen, 1993). These proteins and their homologues recently discovered in wheat (Rosenkrands et al., 1994) are particularly interesting because they are the only proteins of plant origin showing a good level of homology (~30%) with the superfamily of serine protease inhibitors (serpins; Brant et al., 1990). Studies on the physiological role of protein Z_4 have failed to reveal an inhibitory function, and only a weak interaction with chymotrypsin was demonstrated for the 39 kDa barley serpin (Lundgard and Svensson, 1989).

The aim of this work was to further improve the characterization of the major protein of beer, by electrophoretic and immunological techniques, to better understand the structural and functional properties of the major protein, as well as its origin from barley grain proteins.

MATERIALS AND METHODS

Samples. Lager beer samples were purchased from Peroni Industry (Padova, Italy). The malt used for beer production was derived from a mixture of the varieties Alexis, Carina, Gitane, Kaskade, and Triumph.

Barley seeds were obtained from Istituto Sperimentale per la Cerealicoltura (S. Angelo Lodigiano, Milan, Italy). The varieties used in this study were Arda, Alexis, Carina, George, Gitane, Triumph, Kaskade, and three barley samples of unknown origin. The high-lysine mutant Risø 7 (Doll, 1976) was also used (provided by Dr. M. Stanca, Istituto Sperimentale per la Cerealicoltura, Fiorenzuola d'Arda, Piacenza, Italy).

Sample Preparation. Beer protein samples were prepared by $(NH_4)_2SO_4$ precipitation, as previously described (Pressi et al., 1993). The 80% saturation salted-out material was exhaustively dialyzed against deionized water (cutoff, 3.5 kDa) at 4 °C and freeze-dried or stored frozen for subsequent use.

Soluble proteins were extracted from barley grain with distilled water (1 g of ground seeds in 10 mL of water) by stirring for 1 h at 4 °C. After centrifugation, the supernatant

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(supernatant 1) was collected, and the pellet was washed twice with 2 mL of 10 mM Tris-HCl (pH 7.2) by stirring for 1 h at 4 °C. After centrifugation, the supernatant was discarded and the pellet re-extracted with 4 mL of 10 mM Tris-HCl (pH 7.2) containing 1% (v/v) 2-mercaptoethanol (2-ME) by stirring for 1 h at 35 °C. The clear supernatant obtained after centrifugation was then collected (supernatant 2).

An aliquot of both supernatants 1 and 2 was placed in a screw-capped tube and incubated in a boiling water bath for 15 min with occasional mixing. The heavy precipitates formed upon heating were removed by centrifugation, and the clear supernatants were stored frozen for subsequent use.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to Laemmli (1970), utilizing a Mini-Protean II slab cell (Bio-Rad), with a total acrylamide concentration (T) of 12% and containing 2.5% (on T) of cross-linker bisacrylamide.

Samples for protein electrophoresis were prepared by 0.5-fold dilution with 0.6 M Tris-HCl buffer (pH 7.0) containing 40% (w/v) glycerol, 5% (v/v) 2-ME, and variable amounts of the detergent SDS (from 0 to 2%, w/v). After mixing, the various samples were either boiled for 3 min or not, and quantities of 20−40 μL (according to the protein content) were loaded into the slots.

Electrophoresis was carried out at 30 mA/gel until the tracking dye (bromo phenol blue) reached the bottom of the running gel. Gels were stained as described by Koenig et al. (1970) and destained with 7% (v/v) acetic acid or used for the blotting (see Immunoblotting).

Coomassie brilliant blue R-250 stained gels were scanned with an Ultroscan XL apparatus (LKB).

Two-Dimensional Electrophoresis (2-D SDS-PAGE). After performing the first SDS-PAGE separation with extracts not boiled in the presence of SDS, the lane containing the fractionated protein bands was cut from the gel and inserted in a screw-capped tube filled with a 0.2 M Tris-HCl buffer (pH 7.5) containing 1% (w/v) SDS and 1% (v/v) 2-ME. The tube was then incubated in a boiling water bath for 5 min. After cooling at room temperature (R.T.), the gel strip was washed with distilled water and placed in a horizontal position on the top of a second SDS-gel, prepared the same way as that used for the first dimension, but with $T = 18\%$.

Protein Purification. The proteins used for production of antibodies and for sugar content determination were purified by electroendosmotic preparative electrophoresis in the presence of SDS (SDS-EPE). The previously described method of Pressi et al. (1993) was used, except the beer sample was not boiled in the presence of SDS before the electrophoretic fractionation.

Antisera Production. Polyclonal antibodies were raised in rabbits by injecting subcutaneously a total of about 0.2 mg of each of the two purified proteins in SDS-EPE elution buffer diluted with one volume of Freund's complete adjuvant. To denature the immunogens prior to injection, the purified protein preparations were diluted with 0.2 vol of 1.2 M Tris-HCl buffer (pH 7.5) containing 6% (w/v) SDS and 6% (v/v) 2-ME. After mixing, the solution was kept for 5 min in a boiling water bath, cooled to R.T., and immediately diluted with the adjuvant. Four injections were made at 2-week intervals, and 2 weeks after the last injection, the rabbits were bled. Collected blood samples were allowed to clot in an ice-water bath, and sera were separated by low-speed centrifugation. Sera were adjusted to 0.02% (w/v) sodium azide and stored frozen in 20-μL aliquots until used.

Immunoblotting. Immunoblotting experiments were performed as previously described (Curioni et al., 1991) with the exception that the semidry electrotransfer was reduced to 30 min at 10 V. The serum dilution was 1:2000 for both the antibody preparations. Anti-rabbit IgG−alkaline phosphatase conjugate goat antiserum (Sigma) was used as the secondary antibody. Immunoenzymatic activity on blots was revealed with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium.

Glycated Proteins Detection. Sugar-associated proteins were detected after semidry blotting (10 V for 30 min) on polyvinylidene difluoride (PVDF) sheets (Immobilon P transfer membranes, Millipore). The concanavalin A/peroxidase system suggested for glycoproteins by Weiss et al. (1991) was followed, with the modifications that 3% glycoprotein-free bovine serum albumin was used as the blocking agent and peroxidase activity was detected with 3-amino-9-ethylcarbazole and H_2O_2.

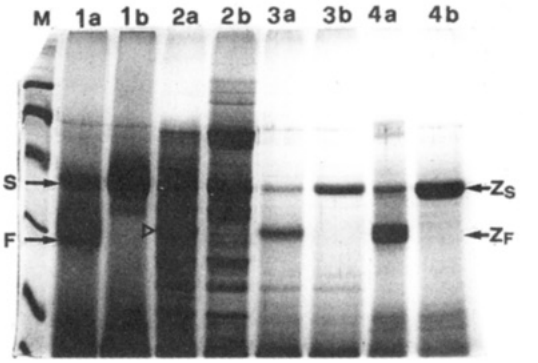

Figure 1. SDS-PAGE results (reducing conditions) for beer proteins (lanes 1), whole water-extracted Risø 7 barley grain proteins (lanes 2), and water-extracted (lanes 3) and 2-ME-extracted (lanes 4) Risø 7 barley grain proteins remaining in solution after boiling (HRP). Samples were either not boiled (lanes a) or boiled (lanes b) in the presence of SDS. The position of FMB (F), SMB (S), Z_F, and Z_S are indicated by arrows. The M_r marker proteins (from top to bottom: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; and trypsin inhibitor, 21.5 kDa) are in lane M. Migration of protein bands is from top to bottom.

Sugar Content Determination. Purified proteins from beer were analyzed for sugar content by the phenol-sulphuric acid method (Dubois et al., 1956), using glucose as the standard. The method was also validated using solutions of ovalbumin of known concentration.

Protein Determination. Protein content was determined by measuring the absorbance at 280 nm, assuming that a protein content of 1 mg/mL gives an absorbance of 1.0. When a more precise measurement was needed, the bicinchoninic acid method (BCA Protein Assay Reagent, Pierce; Smith et al., 1985) was used, with bovine serum albumin as the standard. Samples were incubated for 40 min at 60 °C.

RESULTS AND DISCUSSION

Electrophoretic Characterization. The electrophoretic profile of the salted-out (SO) beer proteins with molecular masses >15 kDa is shown in Figure 1, lane 1b. The pattern corresponds well to that reported by several authors (see for example, Onishi and Proudlove, 1994, and Hejgaard and Kaersgaard, 1983), showing a major component at ~40 kDa, which can be identified as the antigen 1 described by Hejgaard and Kaersgaard (1983). This pattern was obtained after heating the protein mixture in the presence of SDS, which is the classical procedure for protein denaturation prior to SDS-PAGE. On the contrary, when the sample was boiled in the absence of SDS (lane 1a), the protein at 40 kDa was present in relatively lower amounts, and a band with a mobility corresponding to a molecular mass of ~30 kDa appeared as the major component of the electrophoretic pattern. To the best of our knowledge, this band has not been described before, although it can be clearly seen, for example, in the paper published by Dale and Young (1988). These authors did not boil the beer protein solution in the presence of SDS because the effect of a high concentration of urea was considered equivalent to that of heating at 100 °C. A heavy spot appeared at 30 kDa, which was not examined further, being interpreted as due to high molecular weight

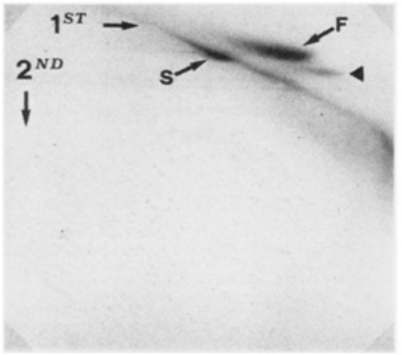

Figure 2. Two-dimensional SDS-PAGE of beer proteins (first dimension, left to right, was not boiled in the presence of SDS; second dimension, top to bottom, was boiled in the presence of 1% SDS). Key: (S) SMB; (F) FMB. The arrowhead indicates the minor 34 kDa band. Total acrylamide concentrations of the first and the second dimension gels were 12 and 18%, respectively.

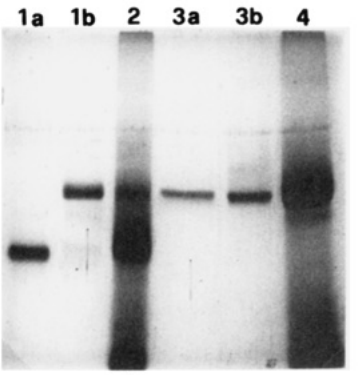

Figure 3. SDS-PAGE results for the purified FMB (lanes 1) and SMB (lanes 3) of beer. Samples were not boiled (lanes a) and boiled (lanes b) in the presence of SDS. Beer proteins that were either not boiled (lane 2) or boiled (lane 4) in the presence of SDS are also shown as reference. Migration of protein bands is from top to bottom.

carbohydrates or nucleic acid polymers that were stained by the silver nitrate (Dale and Young, 1988).

The results of two-dimensional electrophoresis (2-D SDS-PAGE) of the SO beer proteins are shown in Figure 2. The first separation (with 12% *T*) was performed without heating the sample with SDS, whereas the second one (with 18% *T*) was carried out after boiling the 1-D gel strip in the presence of the detergent. The slow moving 40 kDa band (hereafter indicated as SMB) is clearly on the gel diagonal, indicating that no change in mobility has occurred between the first and the second electrophoretic separation. On the contrary, the fast moving 30 kDa band (hereafter indicated as FMB) modified its mobility and shifted to the same level of the SMB, corresponding to an apparent molecular mass of ~40 kDa. This result proves that the mobility of the FMB was converted to about that of the SMB by heating in the presence of SDS. Moreover, no liberation of fragments (at least with a molecular mass >5 kDa, which is the minimum retained in a 18% acrylamide gel) is evident upon denaturation, as indicated by the results from the 2-D gel shown in Figure 2.

An additional minor band of 34 kDa is present in the SDS-PAGE pattern of the fully denaturated beer proteins (Figure 1, lane 1b). This band could be detected with Coomassie blue only when the sample loading was heavy. From the analysis of the gel reported in Figure 2 it is clear that this minor band underwent the same change of relative SDS-PAGE mobility described for the FMB and, in the first electrophoretic separation pattern, it is located in the lower part of the spot corresponding to the FMB.

The FMB and the SMB were then purified by SDS-EPE (Figure 3). Again, when the fractions containing the purified FMB (lane 1a) were heated in the presence of SDS, the band shifted to a level corresponding to that of the SMB (lane 1b), whereas the latter band did not modify its mobility (lanes 3a and 3b). These results confirm those obtained by 2-D SDS-PAGE.

It is important to note that the SDS-PAGE analysis of the purified FMB as it is eluted from the preparative electrophoresis (Figure 3, lane 1a) does not reveal the presence of any band of lower mobility. This means that SMB pre-exists as a distinct molecule in the beer and that it is not derived from the FMB, due to the SDS present during the electrophoresis.

It is thus demonstrated that the SDS-PAGE band corresponding to that which was described by several authors as the main protein of the beer is actually formed by two molecules with the same molecular weight, but showing different electrophoretic mobilities in their incompletely denatured conformation.

It is a common idea that the anionic detergent SDS converts the folded structure of proteins to a rod-like one (Reynolds and Tanford, 1970). Heating the FMB in the presence of SDS is then likely to cause a conformational change in the molecule, involving the acquisition of a less compact molecular shape, that is stabilized by binding with the detergent. This change would result in an increase of the hydrodynamic volume of the unfolded protein and a corresponding decrease of its retardation coefficient. Then, the faster band (FMB) seems to be a compact molecule with respect to the slower one (SMB) that looks like a more relaxed molecule.

The conformational conversion described for the FMB could also be obtained when the sample was incubated at R.T. in the presence of SDS. In this case, the importance of the phenomenon was correlated with the time of incubation as well as with the concentration of SDS present in the beer protein solution. In fact, when the SDS-PAGE analysis was performed immediately after the addition of the detergent, a progressive decrease in the FMB amount was noted from 0.5 to 2% SDS. The decrease was more evident when the same samples were analyzed after 48 or 96 h (not shown). Therefore, the thermal treatment causes only an increase in the rate of conformational transition, and SDS can induce the unfolding of the FMB molecule also in the absence of an energy input, indicating the presence of a relatively low energy barrier. Moreover, the conformational change seems to be a two-state transition because intermediate-mobility bands were always absent. This result suggests that the unfolding involves only one domain of the molecule (Goldenberg, 1990).

An increase in the apparent molecular size upon denaturation has been described for B-hordeins (Doll and Andersen, 1981), gliadins (Singh and Shepherd, 1985), and low molecular weight subunits of glutenin from wheat (Singh et al., 1991). However, in the case of beer protein, the splitting of intramolecular S—S bonds can not be invoked in explaining its unfolding because the omission of the reducing agent from the mixture did not influence the reported results. Therefore, the compact shape of the molecule is maintained by forces of the noncovalent type, such as hydrophobic interactions.

The relative proportion of the SMB and the FMB in three commercial beer samples of different origin was quantified by densitometric scanning of Coomassie blue-stained SDS gels. The ratio between the areas of the peaks corresponding to the SMB and the FMB was higher for one sample (1.5) than for the other two (0.4 and 0.6), indicating a quantitative heterogeneity for the two proteins. It is interesting to note that the sample with the higher ratio showed the best head retention value (HRV a measure of foam stability) of the three, although our data are obviously too limited for any speculation on the correlation between the relative amount of the two bands and the quality characteristics of the foam.

It is well known that the major protein of beer (antigen 1) derives from an albumin present in barley grain, named protein Z. This protein is particularly resistant to proteolysis and maintains its solubility even after drastic thermal treatments (Hejgaard, 1982). Therefore, protein Z passes into the beer while maintaining its molecular size as well as its immunogenicity (Hejgaard, 1982). The water-soluble proteins of barley grain were studied by SDS-PAGE. The sample used for such an experiment belongs to the group of the high-lysine mutants, which were shown to contain a quantity of protein Z higher than that of the normal barley types (Hejigaard and Boisen, 1980). However, the following results, from a qualitative point of view, were the same for the whole set of samples analyzed, including 10 different barley varieties currently used in beer production (Arda, Alexis, Carina, George, Gitane, Kaskade, Triumph plus three varieties of unknown origin; data not shown).

When the water extract was kept in a boiling water bath for 15 min, a heavy precipitate was evident, and the heat resistant proteins (HRP) remaining in solution were examined by SDS-PAGE. The electrophoretic pattern shows, at least in a 12% acrylamide gel, two main bands. The molecular masses of these bands are around 30 and 40 kDa (Figure 1, lane 3a), which corresponds well to the molecular masses of the FMB and SMB present in the beer (compare with lane 1a). These two barley albumins, that will be named Z_F and Z_S, respectively, are related with protein Z, as will be evident from the following results.

When the HRP sample was boiled in the presence of SDS, the Z_F band shifted to a position corresponding to a molecular mass of 40 kDa (Figure 1, lane 3b). This shift resembles the phenomenon described for the SO beer proteins. The electrophoretic pattern obtained with these conditions is very similar to that reported by Hejgaard (1982) for protein Z.

The same electrophoretic behavior was also evident when the whole water-soluble extract was analyzed (Figure 1, lanes 2a and 2b). In fact, although in this case the protein pattern is more complex in comparison with that of the HRP, the disappearance of the Z_F band (lane 2a, empty arrowhead) upon heating the sample in the presence of SDS can be noted along with a reinforcement of the band at 40 kDa (lane 2b). This result was confirmed by 2-D SDS-PAGE experiments (not shown). Therefore, described phenomena were not influenced by the boiling step applied to the solution of barley albumins. The explanation of the reported SDS-PAGE mobility change of the Z_F band upon SDS denaturation is the same given for the FMB of the beer. Moreover, also in the case of the barley proteins, the omission of the reducing agent from the mixture did not influence the obtained results, indicating that the compact shape of the Z_F molecule is not stabilized by intramolecular S—S bonds. The two cysteine residues present in protein Z_4 are, in fact, unlikely to form such a linkage, as deduced from their relative position within the molecular structure (Brant et al., 1990).

The 34 kDa band observed in the beer is absent in the barley albumin solution. It is therefore likely that its formation occurs during the beer manufacture process as a result of a proteolytic cleavage on the Z_F molecule.

The residue deriving from the solubilization of the water-soluble barley proteins was then re-extracted in the presence of 1% 2-ME. The SDS-PAGE analysis of the protein remaining in solution after boiling (Figure 1, lane 4a) shows the occurrence of the two bands Z_S and Z_F, and points out that both the proteins are also present in a bound form, as demonstrated by Hejgaard (1983) for protein Z. Again, the heating of the reductant-extracted protein solution in the presence of SDS caused the modification of the SDS-PAGE mobility of the Z_F band (lane 4b), as previously described for the free form of the protein.

Protein Z has been shown to be a member of the serpins superfamily (Hejgaard et al., 1985). These proteins, most of which play a physiological role in animals, are characterized by the presence of highly conserved internal clusters of hydrophobic residues that are important in determining their typical conformation (Huber and Carrell, 1989), as deduced from the crystallographic structure of α_1-antitrypsin. It also seems that protein Z folds in a similar tertiary structure (Brant et al., 1990). Therefore, it is possible that the SDS-PAGE mobility change observed for the Z_F band under the action of SDS corresponds to the loss of the typical three-dimensional molecular conformation.

Immunological Characterization. The two bands were purified by SDS-EPE with the SO beer proteins not boiled with SDS prior to the preparative electrophoresis. In this way it was possible to use the two purified proteins (Figure 3) as distinct immunogens in two different rabbits. So, two antisera were produced: one against the FMB and one against the SMB.

Our approach involved the use of immunoblotting techniques, in which the high resolution of SDS-PAGE is maintained on the blots, allowing the precise identification of the proteins bound by the antibodies. On the contrary, the immunoelectrophoretic techniques used by Hejgaard (1977) in the characterization of the antigen 1 and its precursors, although useful for quantification of antigens as well as for the determination of their degree of immunological relationship, do not allow the same electrophoretic resolution in terms of band separation that is typical of SDS-PAGE. Therefore, it is possible that the two immunologically related bands (antigens 1a and 1b) identified by Hejgaard (1977) showed the same mobility due to the low resolving power of the electrophoretic system. Moreover, in the present work electrophoretically pure beer proteins were injected as immunogens for the production of antibodies recognizing the barley proteins. This procedure is different than that of Hejgaard and Kaersgaard (1983), who used either an antibody prepared against a protein-rich concentrate of beer macromolecules (called fraction X) or an antibody produced against the purified barley protein Z.

Immunoblotting experiments show that the antibodies produced against the SMB (Figure 4A) recognize

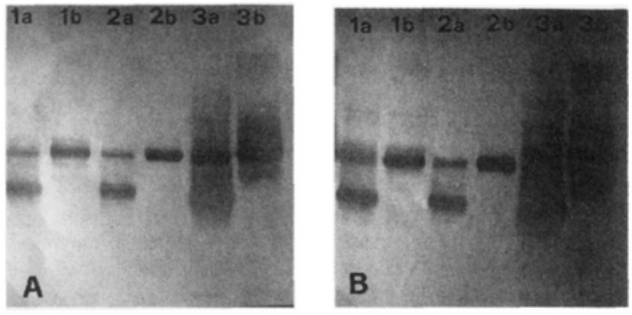

Figure 4. Immunoblotting showing the reaction of the antibodies produced against beer SMB (part A) and FMB (part B), with the whole Risø 7 grain water-extracted proteins (lanes 1), the HRP from Risø 7 grain water-extracted proteins (lanes 2), and the beer proteins (lanes 3). Samples were not boiled (lanes a) and boiled (lanes b) in the presence of SDS. Migration of protein bands is from top to bottom.

both the SMB and the FMB (lane 3a) in the beer and proteins Z_S and Z_F in the barley grain water extract before (lane 1a) and after (lane 2a) ebullition. Again, boiling in the presence of SDS caused the shift of the faster bands at the same level of the slower ones (lanes b). The same results are obtained for the antibodies produced against the FMB (Figure 4B).

The two antibody preparations showed good specificity; that is, no other bands are recognized on the blots. The only exception is the 34 kDa polypeptide present in beer (lanes 3b), confirming its relation with the two major bands and its absence in the barley albumins pattern (lanes 1 and 2).

The reaction of the two antisera with the FMB of beer seems to be fainter than that with the SMB. This fact is only apparent, because the majority of the color at the level of the FMB developed on the back side of the blotting sheet because of the high migration rate of the latter band through the membrane during the electroblotting step. This observation confirms the occurrence of a particularly compact shape for the FMB, compared with the SMB, as indicated earlier. Moreover, the immunostained area on the blot is much more diffuse for the beer sample than for the proteins of the barley grain. This result is probably due to the presence of various close-migrating polypeptides, which share common epitopes with the two main bands. This type of material is absent in the barley albumins; accordingly, its formation would occur during the beer manufacture process. Indeed, the presence of polypeptides carrying minimal modifications of proteolytic origin has been supposed many times (Sørensen and Ottesen, 1978; Hejgaard and Kaersgaard, 1983; Pressi et al., 1993).

The fact that the protein binding pattern is the same for the two antisera, also from a semiquantitative point of view, proves that the two bands are closely related. Identical protein binding patterns also support the speculations we made on the presence of two forms of the same protein, which are characterized by different SDS-PAGE mobility results, stemming from a different molecular conformation.

By testing the antibody binding pattern, it is also demonstrated that the two heat-resistant albumins identified in the barley extract are the actual precursors of the proteins present in the beer. This conclusion is in agreement with the results reported in the literature for protein Z.

Glycated Protein Detection. The concanavalin A/peroxidase method was initially developed for the detection of the enzymatically glycosylated proteins

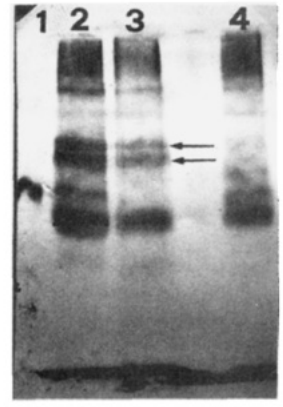

Figure 5. Detection of sugar-associated beer proteins by the concanavalin A/peroxidase method of Weiss et al. (1991). Two beer samples were analyzed under reducing conditions (lanes 2 and 3) and one under non-reducing conditions (lane 4). The arrows indicate the two glycated protein bands appearing after reducing the beer samples. The M_r marker proteins, as in Figure 1, are in lane 1, where the only protein band visible is that belonging to the glycoprotein ovalbumin (45.0 kDa). Migration of protein bands is from top to bottom.

present in the barley grain (Weiss et al., 1991). However, the same system seems to also be useful for the detection of proteins undergoing glycation *via* chemical reactions, as probably occurs in the present case. Only the SMB of the beer is associated with sugars (Figure 5), whereas neither the FMB nor the corresponding barley proteins are glycated. This observation confirms that protein Z does not contain any sugar residue (Hejgaard, 1982). To confirm the results obtained for the beer proteins, the sugar contents of both the SMB and the FMB were determined by the phenol/sulphuric acid method on the EPE purified bands. The SMB contained ~3.3% sugar, an amount similar to that reported by Hejgaard and Kaersgaard (1983) for the beer antigen 1. On the contrary, no sugars were detected in the purified FMB preparation. Previously, Kaersgaard and Hejgaard (1979) reported that about one-third of the antigen 1 was glycated. Hence, this amount can correspond to the quantity of the SMB relative to that of the FMB, which does not carry any sugar residue. In conclusion, only the SMB undergoes glycation during the beer manufacture process, probably as a consequence of Maillard-type reactions (Hejgaard and Kaesgaard, 1983), which are favored by the high temperature treatments. The more open structure of the SMB is likely to be the cause rather than the effect of the presence of sugars associated with the molecule. In fact, this less mobile band is also present (see previous discussion) in the barley grain water-soluble proteins and does not contain any sugar residue, as assessed by the concanavalin A/peroxidase method. These results confirm the findings of Hejgaard (1982). It is then likely that a more open conformation, preexisting in the barley, exposes the reactive sites for the occurrence of the Maillard reaction during the beer manufacture process. This reaction results in the formation of a glycated protein that can be involved in the mechanisms of beer foam stabilization, as suggested by several authors (Bamforth, 1985).

Some other sugar-associated bands were detected by the concanavalin A/peroxidase method in the SDS-PAGE pattern of the beer proteins (Figure 5). In particular, two strong-reacting bands are evident on the blot (arrows), in addition to high molecular weight material streaking on the top of the running gel. It is interesting to note that the two bands indicated by

arrows in Figure 5 were absent when the beer sample was not reduced before the electrophoretic separation (lane 4). Therefore, it seems that they were part of very large disulfide-linked aggregates that could not enter the gel under the described conditions. The presence of this type of aggregate, which would be associated with foam stabilization, has been postulated for a long time (Sørensen and Ottesen, 1978), but direct evidence for their occurrence are, to the best of our knowledge, absent in the literature. A further characterization of the sugar-associated beer proteins is in progress.

GENERAL DISCUSSION

We have demonstrated that what was considered to be the major protein of beer is actually formed by two molecules, which are separable by SDS-PAGE due to their different molecular shapes. The two proteins present in beer are derived, with minor modifications, from two corresponding proteins of the barley endosperm, which share some particular properties. Both are present in a free (water-extractable) and in a bound (2-ME-extractable) form and in increased quantity in a high-lysine barley type; both are resistant to drastic thermal treatments; and both are present in beer, although one is glycated and the other is not. Moreover, they are immunologically related. Finally, they have a similar molecular mass (~40 kDa). All of these characteristics are typical of barley protein Z (Hejgaard, 1982), suggesting that the proteins described in this paper are related to protein Z.

The presence of two different shaped bands sharing common properties as well as an immunological relationship remains to be explained. One possibility would be that they are the product of two different genes. Indeed, the occurrence of other DNA sequences related to the gene coding for protein Z_4 (the gene paz 1 of chromosome 4) has been reported in barley (Brant et al., 1990; Rasmussen, 1993). Moreover, the presence of a polypeptide that is antigenically related but genetically distinct has also been described (Hejgaard, 1984). This polypeptide probably corresponds to the 39 kDa protein, isolated from barley seeds by Lundgard and Svensson (1989), which is similar to protein Z. On the other hand, it seems that protein Z_4 is synthesized by the free and membrane-bound polysomes and initially targeted to alternative sites in the cell (Brant, 1990). This fact indicates a different fate for the same molecule. Thus, an alternative explanation for the presence of two bands with different conformation could be that different posttranslational modifications and folding patterns are possible for the same polypeptide chain. This point needs further clarification, and work is in progress to establish the chromosomal location of the genes coding for the two forms of protein Z described in this paper.

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