E. Steiner and W. Back

A Critical Review of Protein Assays and Further Aspects of New Methods in BrewingScience

Total nitrogen content of barley, malt and beer was usually measured by Kjeldahl method. In brewing science this method has been used for many years to measure total protein (N x 6.25 = protein concentration) in beer, but it measures nitrogen rather than proteins. Kjeldahl method determines total nitrogen but is prone to interference from non-protein and nitrogen-containing compounds, and fails to detect subtle changes in the protein content of wort and beer.
Many quality attributes (e.g. turbidity, mouthfeel, foam stability) and processability (parameters such as filterability, are affected by the protein composition and content in beer. For example protein Z (M_R 40 kDa) [12] is claimed to be responsible for haze formation and LTP1 [25] (M_R 10 kDa) for foam stability. *Siebert* [23] suggests that a higher amount of proline results in a higher turbidity.
Therefore, for brewers its is important to quantitatively measure the protein content and also to qualitative evaluate the protein composition in beer.
The aim of this review is to describe and compare different methods of protein quantification and qualification. For that reason six different methods have been evaluated.

Descriptors: protein assays, Kjeldahl method, Bradford method, lab-on-a-chip analysis, 2D-PAGE, mass spectrometry

1 Introduction

Beer is a complex mixture of over 450 constituents, and, in addition, it contains macromolecules such as proteins, nucleic acids, polysaccharides and lipids [4].

Proteins and protein structure play a major role in beer and beer quality. Beer contains ~500 mg/L of proteinaceous material, including a variety of polypeptides with molecular masses ranging from 5 to 100 kDa the majority of which lie within the 10–40 kDa size range. These polypeptides, which mainly originate from barley, are the product of the proteolytic and chemical modifications during malting and brewing [5, 12, 20, 25].

Proteins influence the whole brewing process not only in the form of enzymes but also in combination with other substances such as polyphenoles. As enzymes they degrade starch, ß-glucanes and proteins, in protein-protein linkages they stabilize foams and are responsible for mouthfeel and flavour stability and in combination with polyphenoles they are thought to form haze. As amino acids, peptides, sal ammoniac they are important nitrogen sources for yeast. Studies on these aspects have already been done for barley (variety differentiation, development of enzymes during germination etc. [2, 9, 10, 27–29]) and for beer. In beer the main focus was on foam and haze active proteins [15, 16, 21, 22].

Authors:
Elisabeth Steiner, Prof. Dr. Werner Back, Lehrstuhl für Brau- und Getränketechnologie, Technische Universität München, Weihenstephaner Steig 20; corresponding author: Elisabeth.Steiner@wzw.tum.de

Figures see Appendix

It is important for brewers to know which methods are most appropriated and useful not only for quantitative but also for qualitative protein assays.

2 Methods

Total protein content (Kjeldahl method, Bradford assay), coagulable nitrogen, nitrogen fractionation (precipitation of magnesium sulfate and phosphomolybdenum acid) and free amino nitrogen of freshly collected beer were immediately measured. Samples of the collected beer were freeze dried and prepared for 2D-PAGE and lab-on-a-chip analysis.

2.1 Kjeldahl method

The standard method for determining protein content of beer is the Kjeldahl method [8, 19]. The standard value of total nitrogen content in beer ranges between 700–800 mg N/L.

Nitrogenous compounds in the beer are digested with hot sulphuric acid in the presence of catalysts to give ammonium sulphate. The digest is made alkaline with sodium hydroxide solution and released ammonia is distilled into an excess of boric acid solution. The ammonia is titrated with standard acid solution.

2.2 Nitrogen fractionation

- Precipitation of magnesium sulfate (> 2.6 kDa). To estimate high molecular weight nitrogen. High molecular weight nitrogen is precipitated with magnesium sulfate and analysed by a Kjeldahl procedure. Standard value: 130–180 mg/L.

- Precipitation of phosphomolybdenum acid (< 2600 Da).: to estimate middle molecular weight nitrogen. Middle molecular weight nitrogen is precipitated with phosphomolybdenum acid and analysed by a Kjeldahl procedure. Standard value: 160–200 mg/L.

2.3 Coagulable nitrogen according to MEBAK [19]. Standard value: 15–25 mg/L

Estimation of high molecular nitrogen. Precipitation of high molecular nitrogen during 5 hours of boiling at 105–108 °C. Digestion of nitrogen with Kjeldahl method.

3 Free amino nitrogen in beer by spectrophotometry (IM) according to EBC [8]

The method gives an estimate of amino acids, ammonia and, in addition, the terminal α-amino nitrogen groups of peptides and proteins. Proline is partially estimated at the wavelength used.

4 Bradford assay

Bradford assay [3] is a protein determination method which involves the binding of Coomassie Brilliant Blue G-250 to protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm which is monitored. This assay is very reproducible and rapid. It is virtually completed in approximately two minutes and presents good colour stability for approximately one hour. There is little or no interference from cations such as sodium or potassium, carbohydrates such as sucrose. The use of strongly alkaline buffers develop a small amount of color but the assay may be run accurately by the use of proper buffer controls.

5 Lab-on-a-chip

Lab on a chip technique capillary electrophoresis was carried out on the Agilent 2100 bioanalyzer [26]. The principles of these electrophoretic assays are based on traditional gel electrophoresis principles that have been transferred to a chip format. The chip accommodates sample wells, gel wells and a well for an external standard (ladder). Micro-channels are fabricated in glass to create interconnected networks among these wells. During chip preparation, micro-channels are filled with a sieving polymer and fluorescence dye. Once wells and channels are filled, the chip becomes an integrated electrical circuit.

Extraction for lab-on-a-chip technique: Resolve 100 mg of freeze dried sample in 1.5 mL of lysis buffer (2M Urea, 15 % Glycerol, 0,1M Tris, pH 8.8, 0.1M DTT). 4 µL of this solution were denatured using 2 µL of Agilent denaturing solution and heated for 5 min. at 100 °C. After dilution with deionised water, 6 µL were applied to the Protein 80+ LabChip (detection performance between 4.5 and 95 kDa) for analysis in the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The ladder consisted of reference proteins of 3.5, 6.5, 15, 28, 46, 63 kDa plus the upper and the lower markers of 95 and 1.6 kDa. According to the Agilent manual any peak detected below 5 kDa is named a system peak and is not included in analysis. Results can be shown in an electropherogram or a gel-like image, as known from SDS-PAGE analysis, where the intensity of bands equals the peak heights in the electropherogram.

6 2D-PAGE

2D-PAGE (Two dimensional polyacrylamide gelelectrophoresis) [11] was carried out on the Ettan™ IPGphor™ 3 IEF System and the Ettan™ DALT*six* Large Vertical System from GE Healthcare on 12.5 % acrylamide gels.

Extraction of the samples for 2D-PAGE was carried out by TCA/ Acetone precipitation [7]:

The combination of TCA and acetone is commonly used to precipitate proteins during sample preparation for 2D electrophoresis, and is more effective than either TCA or acetone alone.

Resuspend 300 mg freeze dried sample in 1 mL TCA 10 % in acetone with 20 mM DTT. Precipitate proteins for at least 45 min at –20 ºC. Pellet proteins by centrifugation (15 min) and wash pellet with 1 mL cold acetone containing 20 mM DTT. Remove residual acetone by air drying or lyophilisation. Resolve the pellet in 0.5 mL lysis puffer (9.5 M urea, 1 % (w/v) dithiothreitol (DTE), 2 % (w/v) CHAPS, 2 % (v/v) carrier ampholytes (pH 3–10) and 10 mM Pefabloc® proteinase inhibitor).

High-resolution two-dimensional electrophoresis (2D PAGE) for the separation of complex protein mixtures is a combination of isoelectric focusing (IEF) in the first dimension in presence of urea, detergents and DTT, with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Proteins are separated according to isoelectric point (pI) and molecular mass (M_R), and quantified according to relative abundance. Depending on the gel size and pH gradient used, 2D PAGE can resolve more than 5.000 proteins simultaneously (~2.000 proteins routinely), and can detect <1 ng of protein per spot. Furthermore, it delivers a map of intact pro-teins, which reflects changes in protein expression level, isoforms or post-translational modifications. This is in contrast to LC-MS/MS based methods, which perform analysis on peptides, where Mr and pI information is lost, and where stable isotope labelling is required for quantitative analysis. An additional strength of 2D PAGE is its capability to study proteins that have undergone some form of post-translational modification (such as phosphorylation, glycosylation or limited proteolysis) and which can be readily located in 2D gels as they appear as distinct 'spot trains' in the horizontal and/or vertical axis of the 2D gel. Thousands of proteins can be resolved in a single experiment allowing the major proteins in a sample to be isolated and protein levels in related samples to be compared. In combination with mass spectrometry, the proteins can also be identified.

In combination with advanced image analysis, 2D-PAGE is a powerful methodology for detecting changes in protein composi-

tion during development, and to pinpoint most influential proteins different processes.

7 Mass spectrometry (MS) [1]

Mass spectrometry is an analytical tool used for measuring the molecular mass of a sample. In proteomics, Matrix Assisted Laser Desorption Ionisation (MALDI) [14] is used for the identification of isolated proteins 2D-PAGE. One method used is peptide mass fingerprinting by means of MALDI-MS. MALDI is based on the bombardment of sample molecules with a laser light to bring about sample ionisation. This procedure usually involves the excision of individual spots from a 2D gel and the enzymatic digestion of proteins within each, before analysing the digest mixture using mass spectrometer. The initial MS spectrum determining the molecular masses of all of the components of the digest mixture, can often provide sufficient information to search a database using just several molecular weights from this peptide map.

With the help of MALDI following analyses and results can be obtained [1]: It is useful to measure accurate molecular weight, to confirm samples, to determine the purity of a sample, to verify amino acid substitutions, to detect post-translational modifications and to calculate the number of disulphide bridges. It is helpful for reaction monitoring of enzyme reactions, chemical modification, protein digestion.

MALDI is needed for amino acid sequencing, confirmation of sequences, de novo characterisation of peptides, identification of proteins by database searching with a sequence "tag" from a proteolytic fragment and also for oligonucleotide sequencing: The characterisation or quality control of oligonucleotides. Even protein folding and protein-ligand complex formation can be monitored and the macromolecular structure can be determined.

8 Results and Discussion

The most applied method in protein determination in beer is Kjeldahl method. Although Kjeldahl method has been automated, it still employs toxic and hazardous reagents. The advantage of this method lies in its suitability, reproducibility and the approval for beer and its raw materials analysis. This method, in combination with a fractionation (precipitation of magnesium sulfate and phosphomolybdenum acid) gives an overview of the amount of different protein fractions in beer. If amino acids and/or free amino nitrogen are analysed additionally a general survey of several nitrogenous components in beer is guaranteed. According to this fractionation information of foam stability (middle-molecular-weight proteins, precipitation of phosphomolybdenum acid), mouthfeel (coagulable nitrogen and nitrogen achieved from precipitation of magnesium sulfate) and fermentation (free amino nitrogen) can be obtained.

The disadvantage of these methods lies in the number of analyses. At least five different methods have to be performed to provide an insight into the protein composition of beer. As a matter of fact not only the quantity but also the duration of these methods implies an error source.

Studies on determination of protein content have already been made [6, 13, 18, 24, 30]. In these articles total protein content and protein composition were measured using SDS-PAGE . In all these articles was indicated that the Bradford assay is recommended for brewing purposes. It is fast, simple, sensitive, reproducible and remarkable lack of response of compoundswhich interfere with other methods. *Siebert and Hii* [13, 24] established, that the Bradford assay is suitable for the detection of high molecular weight proteins, for example foam active proteins.

Several authors compared, for total protein content the Kjeldahl method with the Bradford assay. In all articles it is mentioned that Bradford is more accurate than Kjeldahl method.

Kjeldahl method showed higher nitrogen content as the Bradford assay. With Kjeldahl total nitrogen is determined, using a factor of 6.25, protein content is calculated. It is evident from the "protein" values that most of the measured nitrogen is associated with low molecular weight interfering substances. With the Bradford assay only proteins and not total nitrogen content are analysed. Also, that many proteins are glycosylated and protein assays fail to take account of carbohydrate constituents. This could explain the higher concentration of total protein content in the Kjeldahl assay.

SDS-PAGE requires extraction, gel casting, electrophoretic separation, staining and interpretation. With lab-on-a-chip technique nearly all steps, except for extraction, are achieved in one step. This avoids mistakes and is even faster than 'normal' SDS-PAGE. In figure 1 [17] separation of malt proteins with lab-on-a-chip technique can be seen, this method can be easily applied to beer. lab-on-a-chip technique provides fast and reproducible results which can absolutely be compared with SDS-PAGE. Relative amount of protein can be obtained. Therefore it is possible to compare samples from the same raw material.

In figure 2, proteins were separated with help of 2D-PAGE. In Region B proteins with a molecular weight of ~43 kDa are shown. Proteins of this size are claimed to influence haze formation. Marked spots in 'Region 1' represent foam active proteins.

9 Conclusion

With several different methods, e.g.: Kjeldahl, fractionation, Bradford assay, information on the protein content of beer and some information of their effects on beer quality parameters can be obtained. Bradford assay is easier, faster and cheaper than Kjeldahl method however, it has not been yet established as an analytical procedure in the brewing practice. Bradford assay is recommended for monitoring changes in the protein composition during the brewing process.

To get an insight in protein composition and how proteins influence processability, mouthfeel, foamstability etc., other analyses have to be performed. With the help of a lab-on-a-chip technique a fast overview of several protein components is achieved. To gain

knowledge in protein structure and composition it is necessary to perform 2D-PAGE and MALDI analyses.

10 References

1. 2009, An Introduction to Mass Spectrometry, Astbury Centre for Structural Molecular Biology, Astbury Building, The University of Leeds.

2. Bobalova, J.; Salplachta, J. and Chmelik, J.: Investigation of protein composition of barley by gel electrophoresis and MALDI mass spectrometry with regard to the malting and brewing process. In: Journal of the Institute of Brewing **114** (2008), no. 1, pp. 22-26.

3. Bradford, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. In: Analytical Biochemistry **72** (1976), no. 1-2, pp. 248-254.

4. Briggs, D. E.; Boulton, C. A.; Brookes, P. A. and Stevens, R.: Brewing: science and practice. 1. Cambridge UK/CRC Press: 2004.

5. Curioni, A.; Pressi, G.; Furegon, L. and Peruffo, A. D. B.: Major Proteins of Beer and their Precursors in Barley: Electrophoretic and Immunological Studies. In: Journal of Agricultural and Food Chemistry **43** (1995), no. 10, pp. 2620-2626.

6. Dale, C. J. and Young, T. W.: Fractionation of high molecular weight polypeptides from beer using two dimensional gel electrophoresis. In: Journal of the Institute of Brewing **94** (1988), no. 1, pp. 28-32.

7. Damerval, C.; De Vienne, D.; Zivy, M. and Thiellement, H.: Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. In: Electrophoresis **7** (1986), no. 1, pp. 52-54.

8. EBC: Analytica EBC. Fachverlag Hans Carl: 2007.

9. Finnie, C.; Maeda, K.; Ostergaard, O.; Bak-Jensen, K. S.; Larsen, J. and Svensson, B.: Aspects of the barley seed proteome during development and germination. In: Biochemical Society Transactions **32** (2004), no. 3, pp. 517-519.

10. Finnie, C.; Melchior, S.; Roepstorff, P. and Svensson, B.: Proteome analysis of grain filling and seed maturation in barley. In: Plant Physiology **129** (2002), no. 3, pp. 1308-1319.

11. Görg, A., Lück, C., Weiland, F., Drews, O., Wildgruber, R., Scheibe, B. and Weiss, W.: Two-Dimensional Electrophoresis with Immobilized pH Gradients for Proteome Analysis; 2007; A Laboratory Manual.

12. Hejgaard, J.: Origin of a dominant beer protein immunochemical identity with a beta -amylase-associated protein from barley. In: Journal of the Institute of Brewing **83** (1977), no. 2, pp. 94-96.

13. Hii, V. and Herwig, W. C.: Determination of high molecular weight proteins in beer using Coomassie blue. In: Journal of the American Society of Brewing Chemists **40** (1982), no. 2, pp. 46-50.

14. Hillenkamp, F.; Karas, M; Beavis, R. C. and Chait, B. T.: Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. In: Analytical Chemistry **63** (1991), no. 24, pp. 1193A-1203A.

15. Iimure, T.; Nankaku, N.; Watanabe-Sugimoto, M.; Hirota, N.; Tiansu, Z.; Kihara, M.; Hayashi, K.; Ito, K. and Sato, K.: Identification of novel haze-active beer proteins by proteome analysis. In: Journal of Cereal Science **49** (2009), no. 1, pp. 141-147.

16. Iimure, T.; Takoi, K.; Kaneko, T.; Kihara, M.; Hayashi, K.; Ito, K.; Sato, K. and Takeda, K.: Novel prediction method of beer foam stability using protein Z, barley dimeric alpha -amylase inhibitor-1 (BDAI-1) and yeast thioredoxin. In: Journal of Agricultural and Food Chemistry **56** (2008), no. 18, pp. 8664-8671.

17. Klose, C.; Schehl, B. D. and Arendt, E. K.: Protein changes during barley malting. In: BRAUWELT **148** (2008), no. 36, pp. 1044-1045.

18. Lewis, M. J.; Krumland, S. C. and Muhleman, D. J.: Dye-binding method for measurement of protein in wort and beer. In: Journal of the American Society of Brewing Chemists **38** (1980), no. 2, pp. 37-41.

19. MEBAK: Brautechnische Analysenmethoden. 2nd Volume. 4th Edition. Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission: 2002.

20. Narziß, L.: Abriss der Bierbrauerei. 7. Wiley VCH: 2005.

21. Robinson, L. H.; Evans, D. E.; Kaukovirta-Norja, A.; Vilpola, A.; Aldred, P. and Home, S.: The interaction between malt protein quality and brewing conditions and their impact on beer colloidal stability. In: Technical Quarterly & the MBAA Communicator **41** (2004), no. 4, pp. 353-362.

22. Robinson, L. H.; Healy, P.; Stewart, D. C.; Eglinton, J. K.; Ford, C. M. and Evans, D. E.: The identification of a barley haze active protein that influences beer haze stability: the genetic basis of a barley malt haze active protein. In: Journal of Cereal Science **45** (2007), no. 3, pp. 335-342.

23. Siebert, K. J.: Protein-polyphenol haze in beverages. In: Food Technology (Chicago) **53** (1999), no. 1, pp. 54-57.

24. Siebert, K. J. and Lynn, P. Y.: Comparison of methods for measuring protein in beer. In: Journal of the American Society of Brewing Chemists **63** (2005), no. 4, pp. 163-170.

25. Sorensen, S. B.; Bech, L. M.; Muldbjerg, M.; Beenfeldt, T. and Breddam, K.: Barley lipid transfer protein 1 is involved in beer foam formation. In: Technical Quarterly – Master Brewers Association of the Americas **30** (1993), no. 4, pp. 136-145.

26. Agilent Technologies, 2005, Agilent 2100 Bioanalyzer – 2100 Expert User's Guide, Agilent Technologies, Waldbronn, Germany.

27. Weiss, W.; Postel, W. and Goerg, A.: Barley cultivar discrimination: I. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

and glycoprotein blotting. In: Electrophoresis **12** (1991), no. 5, pp. 323-330.

28. Weiss, W.; Postel, W. and Goerg, A.: Barley cultivar discrimination: II. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing with immobilized pH gradients. In: Electrophoresis **12** (1991), no. 5, pp. 330-337.

29. Weiss, W.; Postel, W. and Goerg, A.: Qualitative and quantitative changes in barley seed protein patterns during the malting process analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with respect to malting quality. In: Electrophoresis **13** (1992), no. 9-10, pp. 787-797.

30. Williams, K. M.; Fox, P. and Marshall, T.: A comparison of protein assays for the determination of the protein concentration of beer. In: Journal of the Institute of Brewing **101** (1995), no. 5, pp. 365-369.

Received 11 May, 2009, accepted 17 June, 2009

Appendix

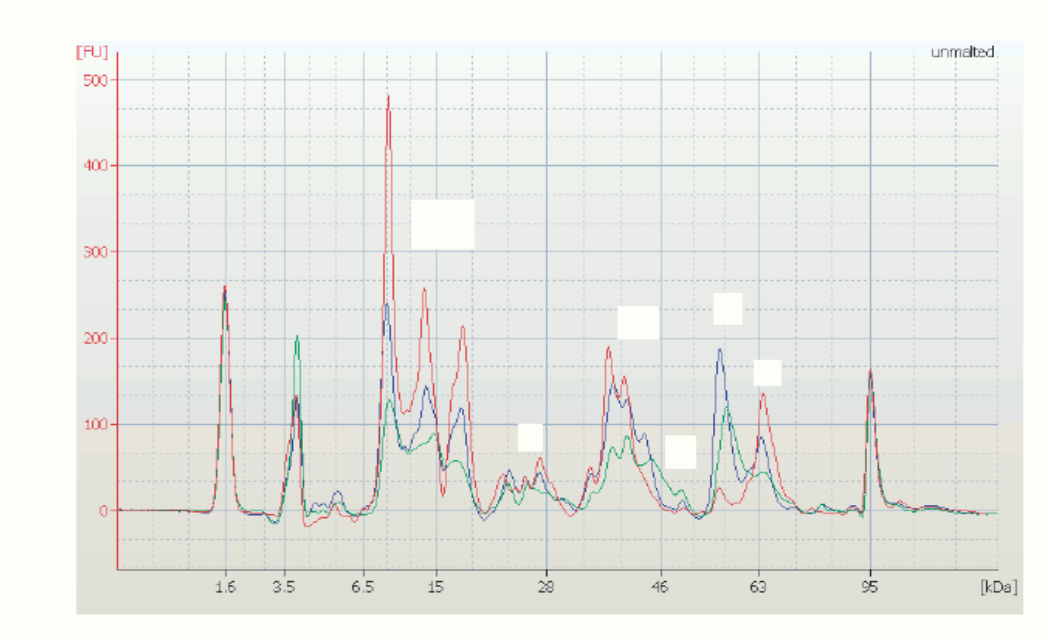

Fig. 1 **Separation of malt proteins with lab-on-a-chip technique [17]**

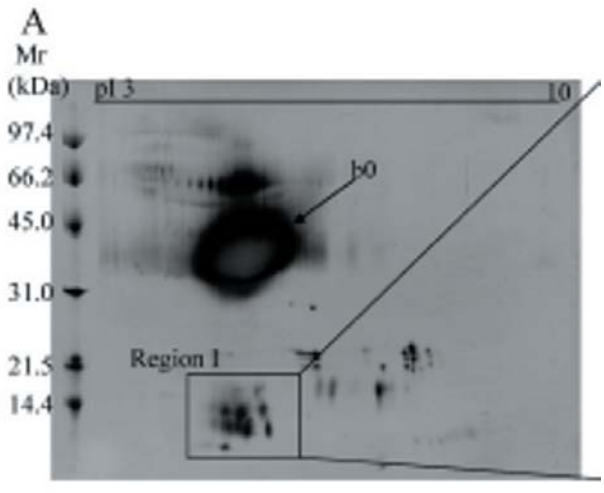

Fig. 2 **2D-PAGE of beer proteins [16]; marked spots in Region 1 represent foam positive proteins**