Analytical Study of Proteolytic Enzymes for Beer Stabilization[1]

J. P. HEBERT, R. SCRIBAN, and B. STROBBEL, *Chaire de Malterie Brasserie, Eaux et Boissons Gazeuses, Ecole Nationale Superieure Des Industries Agricoles et Alimentaires 105, rue de l'Universite F 59509 Douai, France*

ABSTRACT

To optimize beer stabilization the following enzymes were studied: bromelain, ficin, papain, and pepsin. For each enzyme electrophoretic methods on cellulose acetate were used. After electrophoretic separation, chemical and enzymatic determination showed the crude industrial extracts to be highly complex. Refined papain for instance, contained 10 components, bromelain, 13, ficin, 10, and pepsin, 10. Electrophoresis makes it possible to identify the components of a mixture of enzymes and to analyze industrial preparations sold to brewers. Column electrofocusing defines the isoelectric pH of refined papain constituents and peroxidase evaluation is instructive with regard to conditions for harvesting and treating the crude latex from the papaya tree. Although the presence of proteases in beer is easy to determine by estimating residual proteolytic activity, only the use of antipapain and antipepsin immunsera makes it possible to identify the nature of the proteolytic enzyme added to beer, even if it is pasteurized.

Key words: *Beer, Electrophoresis, Immunodiffusion, Papain, Proteases.*

Many processes are available to brewers for improving the colloidal stability of beer. Proteolytic enzymes have been used for over 65 yr, since the registration of patents by Wallerstein in 1911 (32, 33, 34).

In most cases, the protease used is papain. However, its mechanism remains largely unknown and two hypotheses are proposed: hydrolytic cleavage or clotting (8, 9, 11, 28).

In the malting brewing and soft drinks department at Ensia, Scriban and co-workers have been investigating the use of proteolytic enzymes in beer for a number of years. Several enzymes can be used: papain, extracted from the latex of a tropical tree, the *Carica papaya* L.; bromelain, extracted from pineapple; ficin from figs; and pepsin as obtained from gastric juice of pigs.

In order to optimize the colloidal stability of beer, we have attempted to work out a simple electrophoretic method for analysis of industrial enzyme preparations available to brewers.

EXPERIMENTAL

Material

To study preparations containing papain the following samples were tested: 1) crude dried latex in granules or flakes, 2) refined papain known as CFA papain which is a latex purified by physical treatment using centrifugation, filtration and atomization according to Baudart's process, as described by Jones and Mercier (10), 3) twice crystallized papain from Sigma (P 3125), 4) partially purified chymopapain which may contain lysozyme activity Sigma (C 9007), 5) bromelain, from Merck (1651), and 6) ficin an industrial powder, and pepsin an industrial preparation approved by the French Chemical Codex.

Enzymatic preparations sold to the European brewers are in liquid or powder form.

Electrophoretic Methods

Electrophoresis on cellulose acetate.—Zone electrophoresis of 10 μl of a 10% enzyme solution has become for us a standard analytical method for studying enzymatic preparations. It has the advantage of combining simplicity and economy with adequate resolving power.

Experimental conditions for each enzyme were as follows:

Papain
Tris glycin pH 9.3 buffer
μ 0.05 electrophoretic time 1H30 with 200 V at the power supply

Bromelain
Tris EDTA
Na borate pH 8.4 buffer
μ 0.05
1H45
200 V

Ficin
Veronal pH 8.7 buffer
μ 0.05
1H30
100 V

Pepsin
Acetate pH 5.0 buffer
1H15
150 V

Usual staining solutions for protides are used such as nigrosine, amido black and coomassie blue.

Column electrofocusing.—To identify the isoelectric pH of the different components of industrial CFA papain, we applied the electrofocusing technique by using an LKB column (440 ml or 110 ml).

Experiments were conducted in accordance with instructions from LKB, the ampholine supplier, and more specifically according to the application notes (4, 12, 36).

Immunochemical Procedures

These techniques are very sensitive but require the use of immunsera. Immunization of rabbits was obtained by weekly

[1]Presented at the 43rd Annual Meeting, St. Louis, May 1977.

0361-0470/78/000008$03.00/0

intramuscular injections for one month. Solutions administered contained 1–2 mg/ml enzyme emulsified with Freund's adjuvant, in the proportion of 1:1.

The following immunsera were obtained: anti refined CFA papain, anti Sigma papain *sensu stricto*, and anti Sigma chymopapain.

Determination of Proteolytic Activities

For the brewer the problem occurs at two different stages: 1) titration of proteolytic activity, when enzymes are delivered, and 2) survival of enzymes after pasteurization or storage of the beer. For description of the procedures see earlier studies by the authors (22,24).

The reference technique for titration of papain proteolytic activity, registered at the USA Food Chemical Codex, is the one described by the American Pharmaceutical Association (APA). It measures the cleavage of a protein macromolecule, namely casein. This time-consuming and scrupulous procedure requires internal reference samples constituted by papain from the National Formulary (NF). Preliminary titration with Bofors (S 2160) peptide makes it possible to define the quantity of sample required for an official APA titration.

Procedures for proteolytic enzyme detection in beer include the clotting test, the gelatin liquifaction test, the use of Hide Powder Azure (23) and the use of S 2160 (21). The nature of the enzyme involved can only be established by using specific antisera.

Immunodiffusion and electroimmunodiffusion are described by Mancini *et al.* (17) and Loisa *et al.* (16).

Localization of Proteolytic Active Fractions after Analytical Electrophoresis

Proteolytic activity.—After electrophoretic migration, the cellulose acetate is turned upside down on 1% agar gel containing 1% Hammersten casein in a pH 6.0 phosphate buffer with or without activators (0.05 M cystein hydrochloride as reducing agent and 0.05 M EDTA as chelating agent of possible metallic cations). Hydrolysis lasts 1 hr at 5°C. Hemoglobin solution, 0.5%, in a pH 2.5 citrate buffer, may be used instead of casein. In that case, hydrolysis lasts 15 min at 40°C.

Other enzymatic activities.—1) Peptidase activity.—After electrophoretic migration, the cellulose acetate is turned upside down on agar gel. To 100 ml of 1% gel are added: 40 mg L-leucyl-L alanin, 20 mg snake venom (Bothrops Atrox, Sigma V 5375), 40 mg peroxidase (Sigma P 8250) and 75 mg 3-amino-9-ethylcarbazol.

The reaction mechanism is described by Barker (1) and Lewis and Harris (15) and may be summarized as follows:

$$\text{peptide} \xrightarrow{\text{peptidase}} \text{L amino acid}$$

$$\text{L amino acid} + O_2 \xrightarrow[\text{Snake venom is an L aminoacidoxidase}]{} \text{cetoacid} + NH_3 + H_2O_2$$

$$H_2O_2 + \text{3-amino-9-ethylcarbazol reagent} \xrightarrow{\text{peroxidase}} \text{oxidized colored}$$

2) Peroxidase activity.—After the electrophoretic migration the cellulose acetate is steeped in a mixture of 70 ml ethanol and 30 ml pH 4.6 acetate buffer containing 0.25 ml H_2O_2, 30 volumes, as substrate. 100 mg in 100 ml of a potential redox indicator (benzidine or o-dianisidine) gives an ochre color to components with peroxidase activity as described by Delincee and Radola (3).

3) Acid phosphatase activity.—After electrophoresis, the cellulose acetate is steeped in a pH 5.0 acetate buffer, containing α-naphthyl which is then linked to fast garnet, an azodye producing a final orange-red color, according to Wieme (35).

RESULTS AND DISCUSSION

Electrophoresis

A single electrophoresis gives several different figures, depending on detection procedures. The different components fractionated by electrophoresis are stained according to their chemical nature or enzymatic properties. For each type of enzyme a systematic study of buffers and experimental procedures (time, voltage) was carried

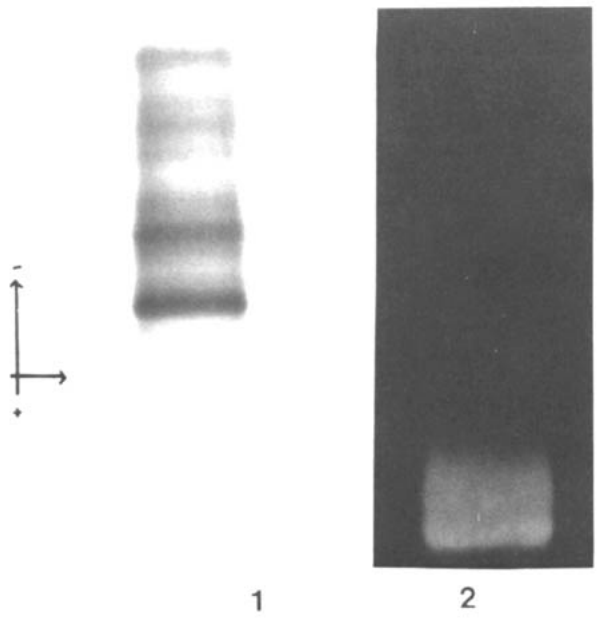

Fig. 1. Cellulose acetate electrophoresis of pepsin Codex 200
Acetate pH 5.0 buffer, 150 V, 1H15
1. nigrosine
2. proteolytic digestion : hemoglobin pH 2.5

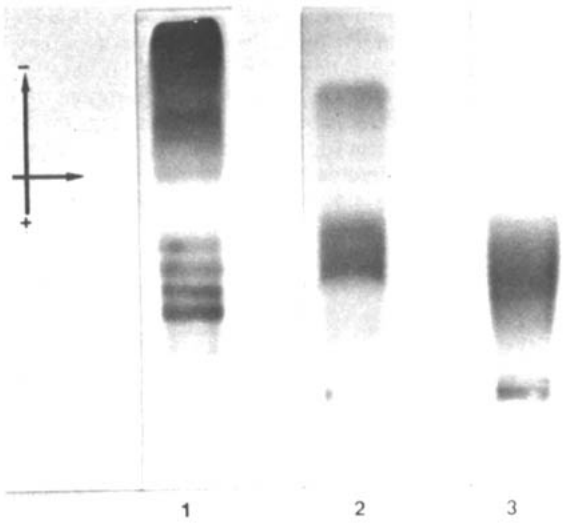

Fig. 2. Cellulose acetate electrophoresis of Merck bromelain
Tris, EDTA, borate pH 8.4 buffer, 200 V, 1H45
1. nigrosine
2. o-dianisidine (peroxidases)
3. Na-α-naphthyl acid phosphate (acid phosphatases)

out in order to ascertain the best possible conditions for cellulose acetate electrophoresis.

Pepsin.—Pepsin, which is of animal origin, clearly has characteristics that distinguish it from proteases of vegetable origin. The electrophoretic study illustrated in Fig. 1 shows 8 different components colored with nigrosine. An acidic fraction, comprising two components, had proteolytic activity revealed by digestion of hemoglobin at pH 2.5 for 15 min at 40°C. Procedures used to detect other enzymatic activities were unsuccessful.

Bromelain.—At pH 8.4 the number of fractions in Fig. 2 shows the great heterogeneity of bromelain. Nigrosine reveals 13 components, 6 of which had proteolytic activity on casein. The use of other staining procedures defines the biochemical nature of 4 other components with phosphatasic acid or peroxidase activity.

Ficin.—Figure 3 clearly illustrates the advantage of using several staining procedures for the same electrophoretic migration. General protein dye enabled 4 fractions to be identified and the fast garnet reagent colored 7 components, 5 of which were not seen with nigrosine. Combination of the dye with enzymatic casein digestion defined two proteolytic components, active at pH 8.7.

One major peroxidase fraction should be noticed. Meanwhile, the proteins colored with fast garnet reagent are difficult to characterize. The esterase nature of these proteins is not proven because the same coloration is obtained without α-naphthyl acetate as substrate.

Papain.—The electrophoregram in Fig. 4 shows 6 protein fractions colored with nigrosine. All of them migrate toward the cathode, indicating that their isoelectric pH values are above 9.3. Enzymatic staining procedures define the nature of these protein components. With casein at a moderate pH of 6.0 the print technique shows a proteolytic digestion for five fractions, one of them being a very basic component. With hemoglobin as substrate at pH 2.5, without activators, only two fractions are revealed, one of which is the papain *stricto sensu.*

Further investigation of this papain led to the appearance of two new peroxidase fractions (Fig. 5). One is acid at the pH of this electrophoresis, *i.e.*, 9.3. Peptidase staining showed three bands.

As described by Barker (1) and Lewis and Harris (15), peptidase resolution comprises reaction between horseradish peroxidase and H_2O_2 as substrate. One of the proteins dyed with peptidase staining is in fact a peroxidase from papain. These two new peptidase components do not correspond to the fraction called peptidase A by Schack (20) and Robinson (19). The pI mentioned by these authors is 11.1 while the migration towards the cathode on electrophoresis at pH 9.3 indicates a pI below 9.3.

According to our column electrofocusing experiment, peptidase A described by Schack (20) and Robinson (19) corresponds to the proteinase D described by Skelton (27).

In conclusion, our analytical procedure on cellulose acetate makes it possible to discriminate 10 different components of an industrial CFA preparation by using only one electrophoretic migration in conjunction with several staining procedures. At pH 9.3, 6 cathodic components, 5 of which hydrolyze casein, are revealed by nigrosine, 2 others are peroxidases, and 2 anodic

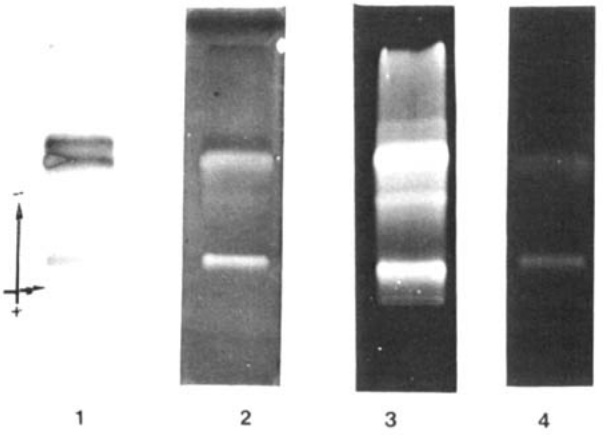

Fig. 4. Cellulose acetate electrophoresis of CFA papain
Tris, glycin pH 9.3, 200 V, 1H30
1. nigrosine
Proteolytic digestion
2. casein pH 6.0
3. casein pH 6.0 + activators
4. hemoglobin pH 2.5

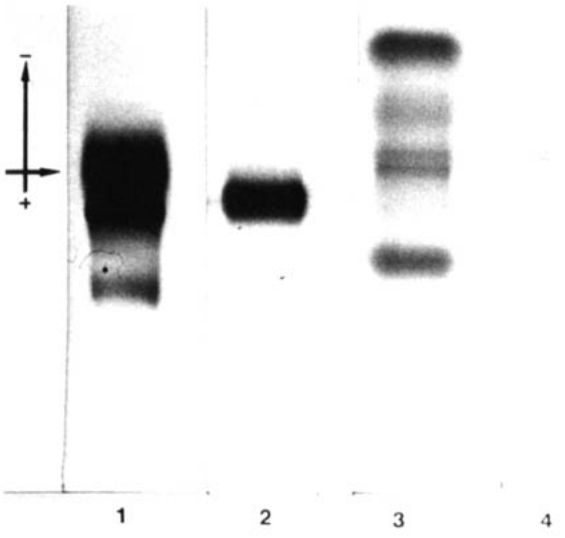

Fig. 3. Cellulose acetate electrophoresis of ficin
Veronal pH 8.7 buffer, 100 V, 1H30
1. nigrosine
2. o-dianisidine (peroxidases)
3. fast garnet
4. peptidases

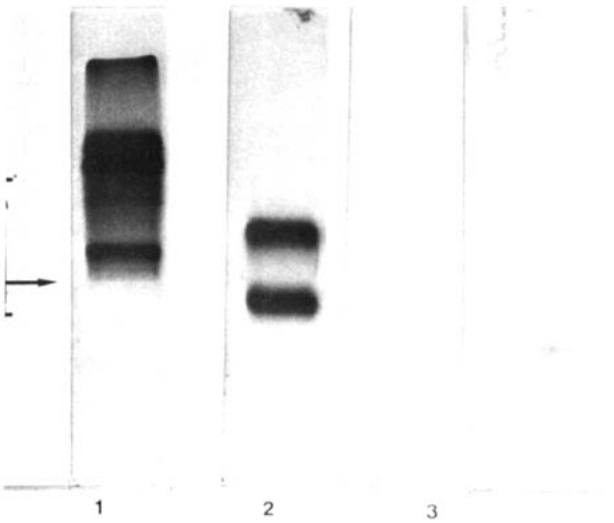

Fig. 5. Cellulose acetate electrophoresis of CFA papain
Tris, glycin pH 9.3 buffer, 200 V, 1H30
Staining of enzymatic activities other than proteolytic
1. nigrosine
2. peroxidases (o-dianisidine)
3. peptidases

fractions are peptidases.

Comparison of our results with those of Schack (20) and Cayle *et al.* (2) is difficult, and raises two problems. First of all, it should be remembered that the harvesting of latex from the papaya tree involves rough procedures such as drying in the sun or with an oven, which makes it difficult to define accurately the product under study. Comparison of electrophoretic pictures is also hazardous because techniques are not identical. Schack (20) used paper as an electrophoresis support, Cayle *et al.* (2) chose acrylamide gel discs, and 5 fractions were revealed. Nicosia (18) used the same support as we did, but under different conditions; only 3 constituents were stained, but the nature of the papain studied was not defined.

Recognition of enzymes in a mixture.—An attempt was made to discover whether a simple electrophoretic method would allow recognition of enzymes in a mixture. We had previously worked out techniques suited to each individual enzyme. To study a mixture, it was necessary to combine several electrophoretic migrations with staining procedures.

Linkage of two electrophoretic procedures, assumed at pH 9.3

TABLE I
Identification of Proteolytic Enzymes in a Mixture

Electrophoresis on Cellulose Acetate

Buffers	Detection Procedures	Results			
Tris Glycin pH 9.3	Nigrosine	every component migrates toward pole (−)	components 1.5 cm 2.5 cm toward pole (−)	one component very acidic one component very basic	
Acetate	*Peroxydase* o-Dianisidine	two components − *one at start line* − one 2 cm toward pole (+)	one component *1 cm toward* pole (−)	one component *remaining at* start line	
pH 5.0	Enzymatic Digestion of Hemoglobin	two components one is above start line *toward pole* (−)		digestion at start line	digestion component *toward pole* (+)
	Conclusion:	Papain	Bromelain	Ficin	Pepsin

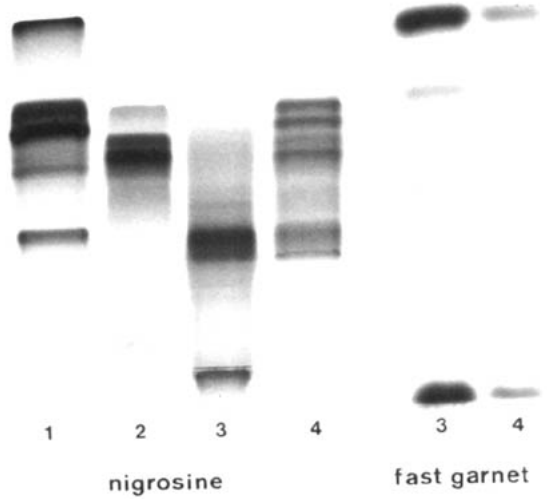

Fig. 6. Identification on cellulose acetate electrophoresis of a mixture of enzymes.
Tris, glycin pH 9.3 buffer, 200 V, 1H30
1. papain
2. bromelain
3. ficin
4. mixture of papain, bromelain, ficin, pepsin

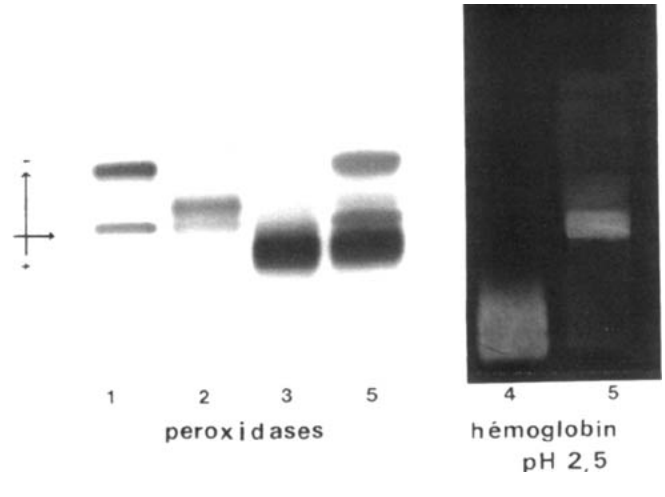

Fig. 7. Identification on cellulose acetate electrophoresis of a mixture of enzymes
Acetate pH 5.0 buffer, 150 V, 1H15
1. papain
2. bromelain
3. ficin
4. pepsin
5. mixture of papain, bromelain, ficin, pepsin

and pH 5.0, permitted recognition of the enzymes in a mixture, as shown in Figs. 6 and 7 and Table I. The same sample is submitted to the two procedures, one in a pH 9.3, tris glycin buffer, the other in a pH 5.0 acetate buffer. The pH 5.0 electrophoresis is disclosed by a hemoglobin hydrolysis which discriminates the pepsin and by peroxidase staining for bromelain detection. This pH 9.3 electrophoresis is revealed by nigrosine and by fast garnet for the phosphatases.

Further Studies of Papain and Commercial Enzymatic Preparations

Because papain is the most widely used proteolytic enzyme, we extended its study and proceeded to analyze crude papaya latex preparations, which constitute the raw material of enzyme purchasers, and, in addition, enzymatic preparations sold to French brewers.

Analysis of papaya latex from different origins.—We studied three deliveries of papaya latex used to make commercial preparations. These three batches included: CFA papain, used as a reference sample; papain latex in granules; and papain latex in flakes. Their characteristics are summarized in Table II.

TABLE II
Characteristics of Papaya Latex

	CFA Papain	Papaya Latex	
		Granules	Flakes
Proteolytic activity APA[a] PU NF	55,800	23,580	49,800
Proteins Kjeldahl % N × 6.25	73.1	65.0	73.1
Neutral Sugars %	1.4	1.9	1.1
Ash	12.0	10.3	11.0
Proteolytic activity per mg protein PU NF	72,560	36,280	68,120

[a]American Pharmaceutical Association.

Although the electrophoretic pictures in Fig. 8 are comparable when a standard dye for proteins such as amidoblack is used, the same does not apply to peroxidase differentiation.

Papain in granules, for instance, comprised no more than one peroxidase fraction colored with benzidine. The results are in agreement with those of Heinicke and Gortner (7) who indicate that peroxidase activity provides information about conditions for harvesting, treating, and storing the crude enzyme. Thus, papain in granules has lost a peroxidase fraction, therefore, has only half the proteolytic activity of papain in flakes. This would indicate that conditions for harvesting and latex preparation were unfavorable.

Analysis of preparations containing papain sold to brewers.—The activities, determined according to the casein digestion procedure described in the Food Chemical Codex, are distributed as follows: from 2220 to 8220 PU NF for powder preparations, and from 4920 to 6480 PU NF for liquid preparations (22). In the case of liquid delivery, electrophoretic study of commercial preparations showed more diffuse fractions than for powder delivery (Fig. 9). Two hypotheses may be formulated.

First, protein autodigestion might occur during storage in the liquid preparation. The fractions thus hydrolyzed would create trails in electrophoretic separation. The second hypothesis concerns the loads, polyalcohols or ionizable molecules used to protect enzymatic proteins delivered in liquid form.

Electrofocusing of CFA papain.—Electrofocusing, or electrophoresis in a continuous pH gradient, appeared some ten years ago. Since then electrofocusing in polyacrylamide gel for the study of beer protides has been developed (5, 6).

Nevertheless, we have not yet obtained striking results for papain; the use of ampholines in the pH 9–11 range raises problems, particularly during gel polymerization. It was necessary to make experiments in a 110 or a 440 ml column.

Electrofocusing confirms the heterogeneity of CFA papain. The figures reported in the literature are lacking in precision. Cayle *et al.* (2) defined crude papain as a mixture of papain, chymopapain, and lysozyme. Skelton (28, 29) introduced a new proteolytic enzyme called "D". Column electrofocusing, presented in Fig. 10 confirms the electrophoregrams and makes it possible to estimate the isoelectric pH collected (Table III).

Recognition of Proteolytic Enzymes in Beer

The problem of detection of enzymatic activity in a treated product is solved by detection of residual enzymatic activity. This is

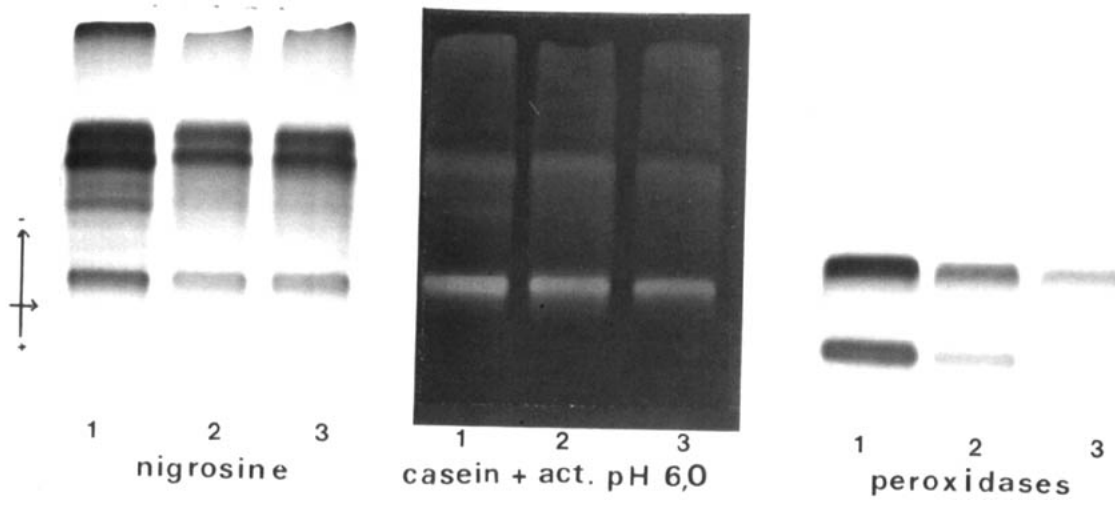

Fig. 8. Cellulose acetate electrophoresis of different papaya latex
Tris, glycin pH 9.3 buffer, 200 V. 1H30
1. refined papain (CFA)
2. latex in flakes
3. latex in granules

only possible if the enzyme remains active and is not completely destroyed during industrial treatment (*e.g.*, pasteurization).

The procedures used by brewers to detect proteolytic enzymes in beer, described in detail elsewhere (25), are briefly reviewed herein. They include what is known as "clotting" of reconstituted buffered skimmed milk. This gives practical information for a daily control of a proteolytic treatment such as during the injection of proteolytic solution after the kieselgur filter. However, the relation between the information provided by the clotting test and the effective proteolytic activity remains uncertain (24). Digestion of the gelatin film sold by the Institut Pasteur Production also gives very simple information (22).

For a more accurate study, titration of residual proteolytic activity is required. This titration is carried out by hydrolyzing a

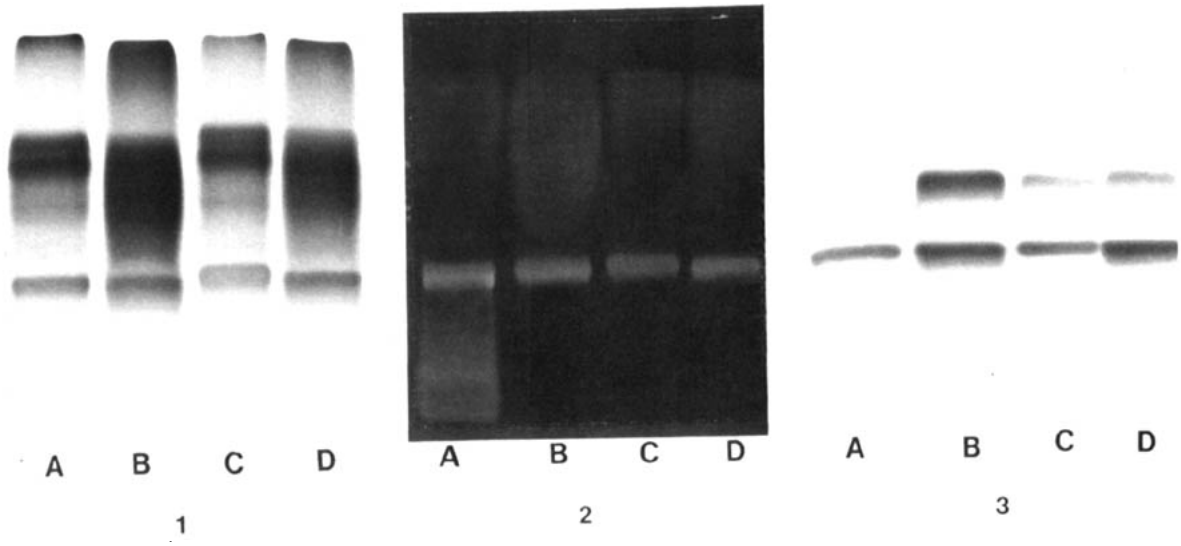

Fig. 9. Cellulose acetate electrophoresis of industrial enzymatic preparations
1. Tris, glycin pH 9.3 buffer, 200 V, 1H30, nigrosine
2. Acetate pH 5.0 buffer, 150 V, 1H15, proteolytic digestion hemoglobin pH 2.5
3. Acetate pH 5.0 buffer, 150 V, 1H15, peroxidases
A.C. Powder preparations
B.D. Liquid preparations

TABLE III
Isoelectric pH of the Major Components of Refined Papain

pI	Identification	Techniques Used for the Identification	Discussion
11.20	lysosyme	lysis of micrococcus	10.5 Smith *et al.* (31)
		2 arcs with immunserum antichymopapain Sigma	Kimmel and Smith (13)
	proteolytic enzyme	casein digestion	11.10 Schack (20), Robinson (19), Skelton (29) peptidase A, proteinase D
10.20	isoenzyme of chymopapain	casein digestion reacts with antichymopapain Sigma immunserum[a]	10.40 Kunimitsu and Yasunobu (14) chymopapain A
10.10	chymopapain	casein digestion reacts with antichymopapain Sigma immunserum[a]	10.10 Kunimitsu and Yasunobu (14) chymopapain B
9.55	peroxidase	reacts with o-dianisidine	original
9.40	papain *stricto sensu*	casein digestion reacts with antipapain Sigma immunserum does not react with antichymopapain Sigma immunserum[a]	8.75 Smith *et al.* (31) 9.60 Sluyterman and De Graaf (30)
<5.0	peroxidase	reacts with o-dianisidine	original

[a]Chymopapain Sigma C_{9007} partially purified may contain some lysozyme activity.

substrate such as Hide Powder Azure or Bofors (S 2160) peptide.

In further studies published in the EBC Proceedings (24, 26), the part played by activators in the proteolytic treatment of beer was shown, as well as the effects of pasteurizing or storage on the residual activity of papain (26, 28).

Immunochemical identification of enzymes.—Although the presence of a proteolytic enzyme in beer is easy to determine by estimating the residual proteolytic activity, the nature of the enzyme involved can only be established by the use of techniques revealing the identity of the molecule itself. Immunochemical procedures are based on the antigenic nature of molecules and are quite suitable for detecting the nature of enzymes used for proteolytic treatment. These procedures are recognized as the most sensitive techniques in the biochemical field but require the preparation of immunsera. The problem is to obtain a valid immunserum. This result depends on a living creature (now and then not very cooperative)—the rabbit. Formation of an immunserum, may require treatment over several one-month immunization periods, during which intramuscular injections are given weekly. After each period, the serum is recovered by bleeding from an ear vein and tested. If suitable for use it is kept. Otherwise treatment must continue for an additional immunization period.

Figures 11 and 12 illustrate how simple immunodiffusion may establish the nature of the enzyme used. The presence of an antigen-antibody complex colored with amidoblack appears to be irrefutable proof of an identity reaction.

Thanks to antipapain and antipepsin immunsera, the nature of the treatment (with papain or pepsin) can be established with certainty.

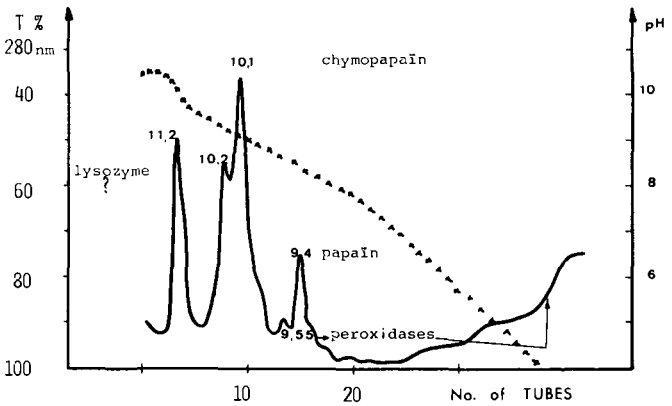

Fig. 10. Electrofocusing diagram of CFA papain
Column electrofocusing 110 ml pH 9-11, 40 mg CFA papain

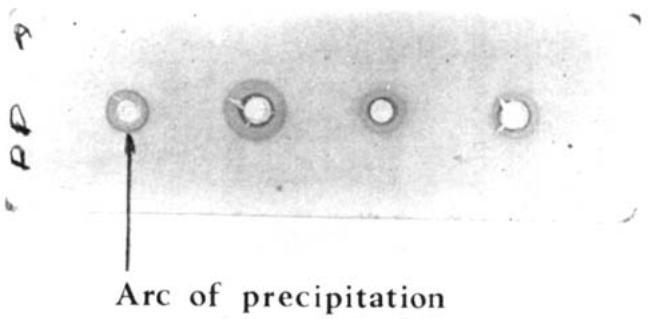

Fig. 11. Identification by immunodiffusion of papain-treated beer
Antipapain immunserum melted in the gelose.
Beers to test in the holes (5 μl).
Antigen-antibody complex colored with amidoblack.

GENERAL CONCLUSION

We worked out a simple analytical technique on cellulose acetate to analyze the different enzymes used in brewing in order to improve the colloidal stability of beer. The study of a mixture of these different enzymes (pepsin, bromelain, ficin, and papain) is then possible by zone electrophoresis.

To optimize in the future the treatment of beers, we continued the study of papain. Peroxidase evaluation by o-dianisidine estimates the treatments undergone by the papaya latex, a crude extract which is the raw material for the enzymatic preparations sold to brewers.

Simple electrophoresis coupled with titration of its proteolytic activity (according to the Food Chemical Codex after pretitration with Bofors (S 2160) peptide) allows definite identification of proteolytic preparations used by brewers.

For accurate estimation more sophisticated immunochemical techniques can definitely prove the use of papain or pepsin treatments to protect beer.

However, the mechanism of action of papain is still not known. Industrial papain is very heterogenous, 10 components revealed by

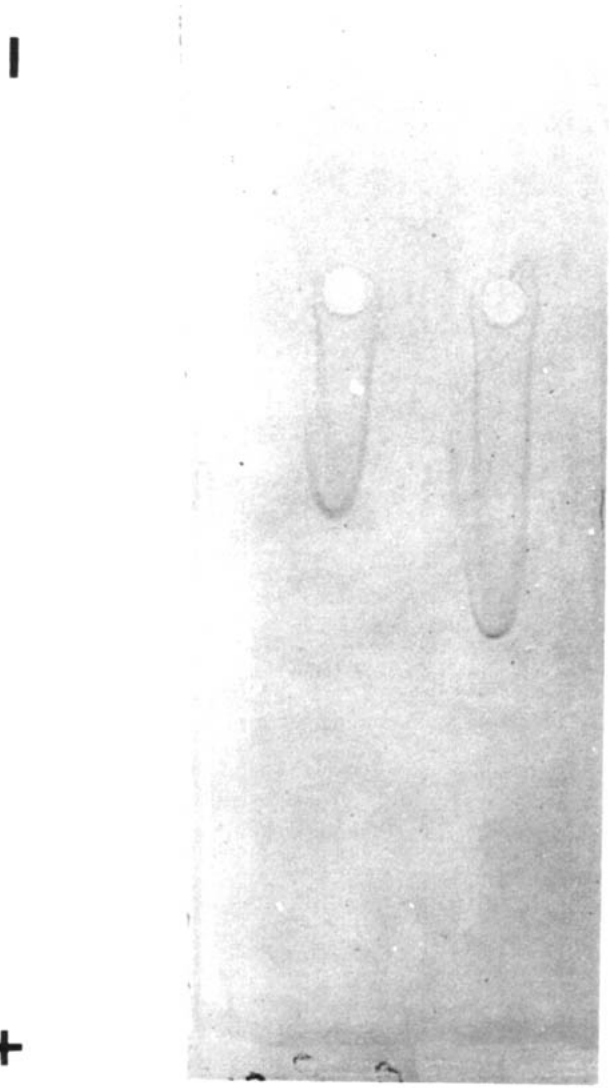

Fig. 12. Identification by electroimmunodiffusion of pepsin-treated beer
Antipepsin immunserum melted in the gelose.
Beers to test in the holes (5 μl).
Antigen-antibody complex colored with amidoblack after electrophoresis.

electrophoresis and enzymatic activities other than proteolytic may play a part in the beer stabilization.

We are continuing our investigation of papain by chromatographic techniques in order to improve our knowledge of its action and to optimize beer stabilization.

Acknowledgment

We are indebted to l'Union Generale de la Brasserie Francaise for financial assistance.

Literature Cited

1. BARKER, J. P. *Biochemical Genetics 12*(3): 199 (1974).
2. CAYLE, Th., SALETAN, L. T., and LOPEZ-RAMOS, B. *Wallerstein Lab. Commun. 27*: 87 (1964).
3. DELINCEE, H., and RADOLA, B. J. *Analytical Biochem. 48*: 536 (1972).
4. HAGLUND, H. *LKB Methods of Biochemical Analysis,* Vol. 19.
5. HEBERT, J. P., and STROBBEL, B. *LKB Application Note* n. 151 (1974).
6. HEBERT, J. P., STROBBEL, B., and SCRIBAN, R. *Bios (7-8)*: 335 (1972); *Bios* (9): 351 (1972).
7. HEINICKE, R. M., and GORTNER, W. A. *Econ. Botany 11*: 225 (1957).
8. HINKEL, E. T., and ZIPPIN, C. *Ann. N. Y. Acad. Sci. 54*: 228 (1951).
9. HORIE, Y. *Amer. Soc. Brew. Chem., Proc. 1964,* p. 174.
10. JONES, J. G., and MERCIER, P. L. *Process Biochem 9*(6): 21 (1974).
11. JONES, M., WOOF, J. B., and PIERCE, J. S. *Amer. Soc. Brew. Chem., Proc. 1967,* p. 14.
12. KARLSSOM, C., and OHMAN, J. *LKB Application Note 16 MLB/an 32* (1972).
13. KIMMEL, J. R., and SMITH, E. L. *Advances in Enzymology 19*: 267 (1957).
14. KUNIMITSU, D. K., and YASUNOBU, K. T. *Methods in Enzymology 19:* 244 (1970).
15. LEWIS, W.H.P., and HARRIS, H. *Nature 215:* 351 (1967).
16. LOISA, M., NUMMI, M., and DAUSSANT, J. *Brauwissenschaft 24*(10): 366 (1971).
17. MANCINI, G., CARBONARA, A. O., and HEREMANS, J. F. *Biochemistry 2:* 235 (1965).
18. NICOSIA, R. *Boll. Chim. Farm. Ital. 113*(7): 405 (1974).
19. ROBINSON, G. W. *Biochemistry 14*(16): 3696 (1975).
20. SCHACK, P. *C.R. Trav. Lab. Carlsberg 36*: 67 (1967).
21. SCRIBAN, R., HEBERT, J. P., and DEVILLERS, G. *Bios 4:* 42 (1976).
22. SCRIBAN, R., HEBERT, J.P., and STROBBEL, B. *Bios 7-8*: 254 (1975).
23. SCRIBAN, R., and STIENNE, M. *J. Inst. Brew. 76*: 243 (1970).
24. SCRIBAN, R., and STIENNE, M. *Eur. Brew. Conv., Proc. Congr. 13th, Estoril, 1971,* p. 393.
25. SCRIBAN, R., STIENNE, M., and STROBBEL, B. *Brasserie 272:* 581 (1969).
26. SCRIBAN, R., STIENNE, M., and STROBBEL, B. *Eur. Brew. Conv., Proc. Congr. 12th, Interlaken, 1969,* p. 397.
27. SKELTON, G. S. *J. Chrom. 35:* 283 (1968).
28. SKELTON, G. S. *Phytochemistry 8:* 57 (1969).
29. SKELTON, G. S. *Enzymologia 40*(3): 170 (1971).
30. SLUYTERMAN, L. A., and DE GRAAF, M. J. *Biochim. Biophys. Acta 258*: 554 (1972).
31. SMITH, E. L., STOCKELL, A., and KIMMEL, J. R. *J. Biol. Chem. 207:* 551 (1954).
32. WALLERSTEIN, L. U.S. Patents 995, 820 (1911); 995, 823-6 (1911).
33. WALLERSTEIN, L. *Proc. Ind. Intern. Brewers Congr. Chicago, 1:* 294 (1911).
34. WALLERSTEIN, L. *Wallerstein Lab. Commun. 24*: 84 (1961).
35. WIEME, R. J. Agar gel electrophoresis, p. 162, Elsevier: Amsterdam (1965).
36. WINTER, A., and KARISSON, C. *Spectra 2000, 29:* 29 (1976).

[Received June 4, 1977.]