Int. J. Chem. Sci. : 2 (3), 2004, 291–298

THE USE OF HPLC FOR THE DETECTION OF LYSOZYME IN DIFFERENT IRANIAN CHEESE SAMPLES

REYHANEH SARIRI

Department of Biology, The University of Guilan, Namjoo St., RASHT, IRAN
Tel: +98 131 3233262, Fax: +98 131 3220912, E–mail: sariri@guilan.ac.ir

ABSTRACT

Lysozyme is a mucolytic enzyme with antibiotic properties discovered by Fleming in 1922. Because of its ability to destroy bacterial cell walls, lysozyme is some times used as a preservative in cheese (E1105). In certain conditions, the use of preservatives is not permitted in cheeses and their presence in food can compromise their quality. In this research, a reverse phase HPLC and a microbial assay were used to quantify small amounts of added lysozyme in some known Iranian pasteurized cheeses. It was found that almost all samples contained the enzyme with a concentration ranging from 12 to 96 ppm.

Keywords: Lysozyme, Cheese, Whey, and HPLC

INTRODUCTION

Lysozyme (EC 3.2.1.17, muramidase) is a mucolytic enzyme with antibiotic properties that was first discovered by Fleming in 1922. Lysozyme was the first protein whose structure was determined by crystallography[1], but until then very little was known about its catalytic properties. The structure of lysozyme has now been studied widely[2,3] and it is one of the mechanistically best understood enzymes. Lysozyme hydrolyzes the β–1,4 linkage between N–acetyl muramic acid and N–acetyl–glucosamine, which occurs in the mucopeptide cell wall structure of certain microorganisms. It, therefore, plays an important role in bacterial disinfection and the defense mechanism. Lysozyme occurs widely in the cells and secretions of vertebrates such as egg whites, and mammalian urine, saliva, tears, milk and kidneys[4], where it functions as a bactericidal agent or help to dispose of bacteria after they have been killed by other means. Lysozyme is a very stable protein with a compact structure which is highly resistant to denaturation, but some chemicals such as sodium dodecyl sulphate, alcohols and fatty acids may inhibit its enzymatic activity.

Due to its antibacterial activity, lysozyme is sometimes used as a preservative in cheese and other dairy products. In certain conditions, the use of preservatives is not permitted in cheeses

Corresponding address:
R. Sariri, Department of Biology, The University of Guilan, Namjoo St., Rasht, Iran, Tel: +98 131 3233262, Fax: +98 131 3220912, E–mail: sariri@guilan.ac.ir

and its presence can compromise the image of the cheese quality. It seems likely that in some circumstances presence of lysozyme in cheese is not shown on its package. Therefore, a suitable method for its detection and quantitation is needed for control purposes. Lysozyme, being a protein, can be determined by high performance liquid chromatography (HPLC) under UV detection at 280 nm. The use of HPLC for detection of lysozyme, however, exhibits some problems that may limit its use. Firstly, as the extinction coefficient of the molecule at this wavelength is relatively low, the analytical technique is not sensitive enough to detect nanograms of lysozyme in 1 mL. Secondly, α–lactalbumin may interfere during HPLC detection of lysozyme in cheese or other dairy products, because the two proteins show structural similarities in many aspects including their molecular weights and tertiary structures. Finally, lysozyme may associate to casein or other proteins present in cheese. A rapid and sensitive reversed phase HPLC has been developed and used for detection of lysozyme in different cheese samples[5]. In this research, a modification of this HPLC method was used to detect the presence of the enzyme in some known Iranian pasteurized cheese samples.

MATERIALS AND METHODS

All chemicals were purchased from Merck Chemical Company, Darmstadt, Germany. They were HPLC grade and used without further purification. Pasteurized cheese samples were obtained from different cheese and dairy product factories. Hen egg white lysozyme to be used as standard was purchased from Sigma (code number L–6876 for laboratory use). The raw milk (2.5% fat) needed for making cheese in the laboratory, was also purchased from a local dairy factory.

Preparation of semi–hard cheese

Laboratory prepared cheese samples were made by mixing one liter of raw milk with rennet and natural whey culture using a modification of cheese manufacturing procedure given by Corradini[6]. Approximately 2 mL aqueous solutions of lysozyme were added to each batch so that the final concentration of the enzyme in each batch varied between 0–60 ppm. Each batch was heated and the cheese was moulded in plastic moulds at 30°C for 72 h after coagulation. The pH was reduced to about 5.0 and the cheese was then salted and ripened at 20°C for two weeks. The whey and cheese were weighed and kept refrigerated for further experiments. Fifteen semi–hard pasteurized packed cheese samples were obtained directly from their factory. Lysozyme was extracted from these cheese samples as well as cheese and whey produced in our laboratory using the following procedure.

Extraction of lysozyme from cheese samples

Finely grated (< 200 mesh) cheese (1.0 g) was sampled according to procedure given by International Dairy Federation (IDF)[7] and added to NaCl 1M (30 mL) using a magnetic stirrer. The pH of the solution was adjusted to 6.0 using 1M NaCl, stirring continued for at least 30 min

at 37°C and the semi–homogenized solution kept under magnetic stirring for 90 min at room temperature until a fully homogenized solution was obtained. A solution of 1M HCl was added carefully until the pH reached 4.5. The extract was filtered using a 0.22 µm membrane after 30 min remaining at room temperature and 40 µL injected directly onto HPLC column.

Extraction of lysozyme from whey

Cheese whey (5.0 mL) was added to NaCl 1M (25 mL) using a magnetic stirrer. The pH of the solution was adjusted to 6.0 using 1M NaCl, stirring continued for 20 min at 37°C and the homogenized solution kept under magnetic stirring for 75 min at room temperature until a fully homogenized solution was obtained. A solution of 1M HCl was added carefully until the pH reached 2.5. The extract was filtered using a 0.22 µm membrane after 30 min remaining at room temperature and 40 µL injected directly onto HPLC column.

Both extracts from cheese and whey samples were kept refrigerated for lysozyme analysis. The presence of lysozyme in extracts was then detected by both HPLC and microbial methods.

Biological activity of lysozyme in cheese extracts

The biological activity of lysozyme was measured in cheese and whey extract by a microbial assay based on the decrease in turbidity (A580) following lysis of a suspension of *micrococcus luteus (micrococcus lysodeikticus)*, the substrate for the enzymatic action of lysozyme[8-11]. We had previously used a modification of this technique to measure the activity of lysozyme extracted from *in–vitro* and *in–vivo* spoiled contact lenses[12].

Detection of lysozyme in cheese and whey extracts by HPLC

Lysozyme was detected in extracts by reverse phase HPLC, a modification of the method described by Herraiz[13]. The specific HPLC conditions used in this work were as follows: The column was a reversed–phase polyethylene column 250 x 4.6 mm, 300 Å pore size and 4 µm particle size, the two elution solvents A and B (Table 1) with different proportions at different elution times, HPLC run time was 35 min, column temperature 50°C, injected volume 40 µL and the flow rate 1mL/min. The elution conditions are given in Table 2 as proportion of solvent A. The detector was a UV type spectrophotometer and the absorbance at 280 nm was recorded in each case. The quantity of lysozyme was calculated from the area between two valleys using a calibration curve obtained from standard lysozyme solutions. A stock 1 mg/mL lysozyme solution was prepared and diluted to give 0.5–10 µg/mL standard solutions. 20 µL of the standard lysozyme solution was injected and HPLC under gradient elution was run. Total concentration of lysozyme in standard solutions was determined by measuring absorbance (280 nm) of eluted fraction after 5 minutes and using the specific extinction coefficient[14].

Table 1. The composition of eluting solutions A and B, prepared from equal proportion of each component

Solvent Mixture	Water + 0.1% Trichloroacetic acid	Acetonitrile + 0.1% Trichloroacetic acid
A	100 g	50 g
B	0	150 g

Table 2. Eluting conditions at different elution times expressed as proportion of solvent A, the flow rate was 1 mL/min, the total run–to–run time 40 min, column temperature 37°C and injected volume 40 μL

Proportion of A (%)	Range of elution time (min)
100	0–20
100–50	20–21
50	21–22
50–100	22–23
100	23–35
100	35–40

RESULTS AND DISCUSSION

To obtain a calibration curve, the height of UV response at 280 nm was plotted against concentrations of standard lysozyme solutions. The reverse phase HPLC was performed on extracted solutions[13]. It is well known that α–lactalbumin is one of the most important milk proteins with molecular weight, hydrophobicity, amino acid sequence and many other characteristics similar to lysozyme[15].

Figure 1 shows the HPLC chromatogram under gradient elution and UV detection of a pure lysozyme solution and the acid whey obtained from milk. It can be seen that lysozyme was eluted

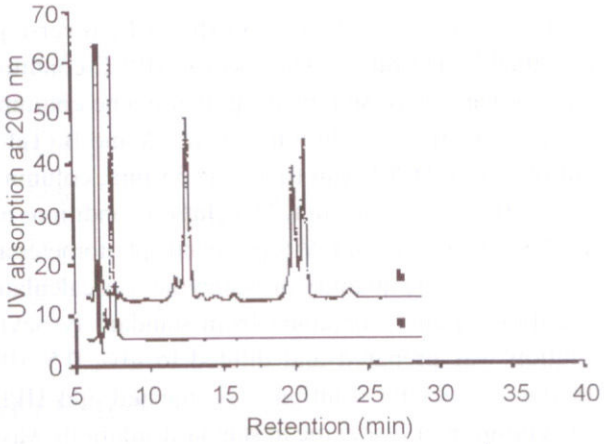

Figure 1 The HPLC chromatogram under gradient elution and UV detection for (a) pure lysozyme and (b) lysozyme and other proteins remained in whey after cheese making. α–lactalbumin is eluted at about 12 min while other milk proteins are eluted in a relatively long time

after about 5 minutes under gradient elution conditions while the retention time for α–lactalbumin was about 12 minutes under the same conditions. The injected volume for the standard lysozyme solutions, cheese and whey extracts was 20 µL, 80 and 100 µL respectively. Using the extraction techniques described in experimental section, lysozyme was completely desorbed from casein and other milk proteins. Approximately 1 g lysozyme was extracted using 25 mL high concentration (1M) of extraction solution (NaCl with a pH near neutral). Table 3 shows a comparison between the quantity of lysozyme in different cheese samples determined by HPLC and *micrococcus lysodeikticus* method. To keep privacy, the brand names of cheese samples have been omitted. All commercial samples were semi–hard pasteurized cheese with similar (2.5%) fat that were provided by their manufacturers as a gift. Samples 1–5 are the semi–hard cheese prepared in the laboratory to which 20, 30, 40, 50 and 60 ppm lysozyme had been added, respectively. Although only five commercial cheese samples are presented in this table (samples 1–5 are the laboratory prepared cheese samples), but the results are combined so that the similar results have been omitted. It is apparent from Table 3 that the amounts of lysozyme measured by HPLC method are 10–25% higher than values obtained from microbial assessment in all cases. This discrepancy may be explained by two facts, firstly the reverse phase HPLC method used here is more sensitive than the microbial assay, and secondly some enzyme activity may have been lost during the extraction procedure. It should be beard in mind that the *micrococcus lysodeikticus* method determines biological activity of lysozyme, while the amount measured by HPLC is the total enzyme present in the extracts. The quantity of lysozyme in whey is very low which indicates complete desorption of lysozyme from other milk proteins and its complete presence in cheese.

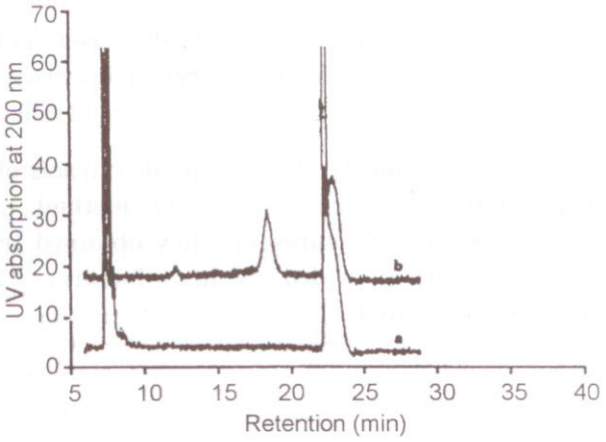

Figure 2. Chromatogram obtained under HPLC conditions given in the text. The injected volume was half of the amount used for Figure 1. (a) mixture of standard lysozyme and a–lactalbumin and (b) lysozyme and other proteins remained in whey after cheese making. The peak in about 4 minutes retention time is due to the soluble molecules usually present in milk and dairy products (proteose peptones)

It has been shown that chromatograms obtained by reverse phase HPLC for all commercial preparations of egg white lysozyme are comparable[5]. This finding was used in this research as the basis for detection of added lysozyme in dairy products. The HPLC results obtained for cheese samples of different manufacturers, not declared to contain lysozyme, showed that the enzyme was present in almost all samples more than expected. Although the use of lysozyme in

cheese making is necessary to prevent late blowing by hindering growth of *clostridium tyrobutyricum*[16], its presence in food may some times compromise the image of quality perceived by consumers. This can be the reason for not declaring lysozyme in cheese and other dairy products. The methods described in this study are suitable for detection and quantification of small amounts of the enzyme in cheese and milk.

Table 3. The quantity of lysozyme in extracts of various cheese samples determined by HPLC and *micrococcus lysodeikticus* method. Lab 1–5 are laboratory prepared cheese samples, whey 1–5 represent whey obtained from those cheeses, commercial 1–5 are cheese samples provided by manufacturers. The similar data between commercial samples are omitted

Cheese sample	Lysozyme in the extraction solution (ppm)	
	HPLC	*micrococcus lysodeikticus*
Lab 1	17.4	15.6
Lab 2	26.8	28.8
Lab 3	35.5	37.2
Lab 4	46.8	47.9
Lab 5	54.1	57.2
Whey 1	2.8	3.0
Whey 2	1.7	1.9
Whey 3	3.1	3.4
Whey 4	2.5	2.9
Whey 5	3.4	3.7
Commercial 1	62.8	74.1
Commercial 2	54.6	66.6
Commercial 3	53.8	41.5
Commercial 4	38.9	34.4
Commercial 5	32.7	23.2

CONCLUSION

Based on the results obtained from this study, it can be concluded that the presence of even small amount of lysozyme in dairy products can compromise the quality of dairy products. Therefore, a sensitive chromatographic method such as reverse phase HPLC described here is needed to detect undeclared lysozyme in cheese and dairy products. The method described in

this study is a fast, reliable and sensitive way to detect very low amounts of enzyme (in the range of ppm). It can easily be used for routine analyses and control of dairy products. The microbial assay of the enzyme is also a very specific and reliable technique that can be used for precise detection of lysozyme present in higher amounts.

REFERENCES

1. C. C. Blake, D. F. Koenig, G. A. Mair, A. C. North, D. C. Philips and V. R. Sarma, Structure of hen egg–white lysozyme. Nature, **206**: 757, (1965).
2. M. Buck, J. Boyd, C. Redfield, D. A. Mac Kenzie, D. J. Jeens, D. B. Archer and C. M. Dobson, Structural determinants of protein dynamics: analysis of 15N NMR relaxation measurements for main–chain and side–chain nuclei of hen egg white lysozyme. Biochemistry, **34**, 4041, (1995a).
3. M. Buch, H. Schwalbe and C. M. Dobson, Characterization of conformational preferences in a partly folded protein by heteronuclear NMR spectroscopy: assignment and secondary structure analysis of hen egg white lysozyme in trifluoroethanol, Biochemistry, **34**, 13219, (1995b).
4. T. D. Drurance, J. S. Int. Sim, and Nakai, Egg uses and processing technologies, New developments. Wallingford UK: CAB International, 77 (1994).
5. L. Pellegrino and L. A. Tirelli. A sensitive HPLC method to detect hen's egg white lysozyme in milk and dairy products, International Dairy J., **10**, 435 (2000).
6. C. Corradini and F. P. Fox, Cheese: Chemistry, physics and microbiology, Chapman & Hall: London, U.K. 2 (1993). p. 221
7. I. D. F., Milk and milk products, Guidance on sampling, International Dairy Federation, Brussels, Belgium, International Standard. 50C (1995).
8. W. W. Gosnell, M. Kroger and F. Katz, Improvement on the agar plate method to determine lysozyme. J. Milk Food Technology, **38**, (1975).
9. I. Lie, M. Syed and H. Solub, Improved agar plate assays of bovine lysozyme and haemolytic complement activity, Acta Veterania Scandinavia, **27**, (1986).
10. G. Mucchetti, E. Neviani and A. Daghetta, Determinazione turbidimetrica dell'attivita del lisozima in latte, formaggio e siero. Scienza e Tecnica Lattiero–Casearia, **41**, (1990).
11. A. H. McKenize and F. H. White, Determination of lysozyme activity at low levels with emphasize on the milk enzyme, Analytical Biochemistry, **157**, 367 (1986).
12. R. Sariri, Interaction of tear proteins with hydrogel contact lenses, Ph.D. thesis, Aston University, UK. (1995). p. 191

13. T. V. Herraiz, Casal and M. C. Polo, Reversed–phase HPLC analysis of peptides in dairy samples, Z. Lebensm Unters Forsch. **199**, 265 (1994).

14. N. Y. Farkin, Indigenous enzymes in milk. In P.F. Fox, Advanced Dairy Chemistry–1: Proteins, Elsevier Applied Science UK. (1992). p. 339

15. H. A. McKenzie and F. H. White. Lysozyme and α–lactalbumin structures, functions and interrelationships, Advances in Protein Chemistry, **41**, 173 (1991).

16. IDF, The use of lysozyme in the preventation of late blowing in cheese, Brussels, Belgium: International Dairy Federation, Buletine, (1987) 50C.

Accepted : 3.5.04