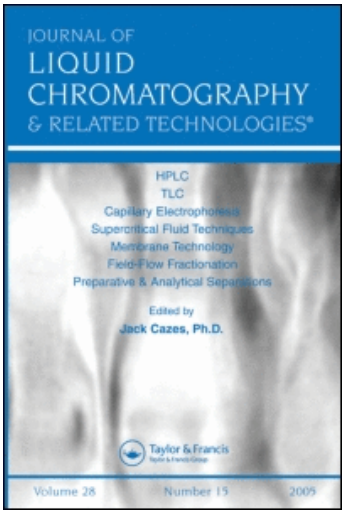

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Effect of Temperature Variation on Metallothionein Sub-Isoform Separation by Reverse Phase High Performance Liquid Chromatography

G. Bordin[a]; F. Cordeiro Raposo[a]; A. R. Rodriguez[a]
[a] European Commission Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg, Belgium

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EFFECT OF TEMPERATURE VARIATION ON METALLOTHIONEIN SUB-ISOFORM SEPARATION BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

G. Bordin, F. Cordeiro Raposo, A. R. Rodriguez

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg
B-2440 Geel, Belgium

ABSTRACT

Sub-isoforms of rabbit liver metallothionein (MT-RL) and of its two main isoforms (RL-1 and RL-2) have been readily separated at room temperature on a reverse phase column using a gradient of TFA and acetonitrile. MT samples were prepared at neutral and acidic pH. The detection was double, consisting of two UV and electrochemical (EC) detectors on line, the EC (coulometric) detection being based on the oxidation of the thiol groups into disulphides at the graphite electrode. Two types of peaks were observed, those equally detected by UV and by EC, being attributed to original thiol containing sub-isoforms and those less hydrophobic, detected by UV but hardly at all by EC, being attributed to oxidised species containing disulphides instead of thiols, hence not detectable in our EC mode. All peaks in RL could be attributed to either RL-1 or RL-2 contribution, except for one of the oxidised forms. The elutions were then carried out at various temperatures between 25 and 60°C.

The temperature appears to have two main effects on MT sub-isoform separation: moderate enthalpy changes (-1 to -2 kcal.mol^{-1}) represented on the chromatograms by small retention time decreases with increasing temperature; important effect on peak "detectability": when the temperature rises, a clear alteration of the oxidised peaks is observed, especially above 50°C. As a result, between 20 and 40°C all peaks are perfectly detected and resolved, while at 60°C only those peaks assumed to be the original sub-isoforms, hence heat-stable, are present.

INTRODUCTION

It is known that metallothioneins (MT), sulphydryl-rich proteins with a selective capacity to bind metal ions,[1-4] exhibit a highly variable polymorphism. Besides the two main isoforms found in mammals, MT-1 and MT-2 separated by anion-exchange chromatography, each MT isoform often contains several sub-isoforms having potentially different biological significance. It is therefore of primary importance to have analytical techniques available which can provide high resolution efficiency. Different methods using high performance liquid chromatography HPLC with various modes of detection[5-9] or capillary electrophoresis CE[10-14] have been employed to show the large extent of MT microheterogeneity.

In a previous paper,[15] we showed, for the first time, the usefulness of using reverse phase HPLC with on-line UV and electrochemical (EC, coulometric) detectors to characterise MT sub-isoforms of mammalian origin (horse kidney and rabbit liver). The electrochemical detection is based on the thiol oxidation at a porous graphite electrode according to the reaction 2 R-SH $\rightarrow$ RSSR + 2 H^+ + 2e^-.

With this system it was possible to discriminate putative original sub-isoforms containing only reduced thiols (equivalent UV and EC responses) from the chemically oxidised forms containing no more or only very few remaining SH groups (good UV but lack of or low EC signals). In the same work, the effects of the MT sample preparation pH, acid or neutral, as well as those of storage time were also investigated.

Generally speaking, the column temperature is another important factor in gradient separation of proteins on reverse phase HPLC. It has already been observed that an increase of temperature very often leads to a reduction of peptide or protein retention times and also to a better resolution.[16-20] Therefore, in an attempt to improve our previous separations, we have presently studied

the effect of column temperature on the retention of the various sub-isoforms of the MT from rabbit liver (RL) and of its two isoforms (RL-1 and RL-2). To our knowledge, the role of this parameter on the MT isoform separation by HPLC has never been investigated *per se*.

We will see that apart from the expected changes in capacity factors, the whole chromatograms are affected by the temperature variations. The following statements should be remembered: an increase in temperature often provokes the denaturation of many proteins, however metallothioneins are heat-stable proteins; in RP-HPLC, the hydrophobicity of the stationary phase and/or that of the organic solvent used for elution might be the main cause of denaturation of proteins due to the breaking of hydrophobic interactions which maintain their original conformation.

EXPERIMENTAL

HPLC Instrumentation and Procedure

The chromatographic system consisted of a Kontron chromatograph (Zürich, CH) equipped with a dual piston pump Model 420, a gradient-former GF 425, and a HPLC 360 autosampler with an injection loop of 100 µL. The separations were performed on a reverse phase column Hi-Pore RP 318 (250 x 4.6 mm), 300 Å pore size and 5 µm particle size (Bio-Rad Laboratories), with the following elution programme: linear gradient 10-25 % B in 50 minutes, at a flow rate of 1 $mL.min^{-1}$, where A was 0.1 % (v/v) trifluoroacetic acid TFA and B was 0.1 % (v/v) TFA in acetonitrile. The column was kept in an Alltech 330 Column heater in order to control the separation temperature. The detection system included a UV capillary detector Model 433 (Kontron) set at 230 nm followed by a Coulochem Model 5100 A electrochemical detector (ESA Inc., Bedford, USA) set at a potential of E_2 = 0.6 V used with a Model 5020 guard cell (E = 0.65 V) and a Model 5011 analytical cell containing two coulometrically efficient porous graphite working electrodes (E_1 and E_2). All metallothionein solutions were prepared at a concentration of 600 $mg.L^{-1}$.

Chemical

Metallothioneins of rabbit liver Cd, Zn MT (RL, lot 23H9550) and its purified isoforms MT-1 (RL-1, lot 94H9504) and MT-2 (RL-2, lot 90H9605) and TFA (1 mL ampoules) were purchased from Sigma (St Louis, MO,USA).

MT solutions were prepared at neutral (water) and acidic (TFA) pH. Acetonitrile was HPLC grade (Super gradient grade, Lab-Scan, Dublin, IRL). Ultra pure water was obtained from a Millipore Mill-RO 10 Plus deionisation system followed by a Milli-Quater system (18 MΩcm resistivity) and a sub-boiling quartz distillation unit (Quartex SA, Paris, F).

The mobile phases were filtered through 0.22 μm Millipore membranes and continually purged with helium to remove dissolved oxygen.

NB: In this article, peak numbering matches increasing retention time t_R and labelling such as H_1 stands for the height of peak 1.

RESULTS

Effect of Temperature on RL-1 Sub-Isoform Separation

The MT RL-1 was firstly prepared in neutral pH and eluted at 25°C. The polymorphism observed under these conditions (Figure 1a-a') is similar to our previous results:[15] four peaks, labelled from 1 to 4, detected by both UV and EC (38 min < t_R < 46 min) and attributed to putative original sub-isoforms and at least four other peaks, denominated α, β, γ and ω and eluted earlier (t_R < 38 min), largely detected by UV but only slightly by EC. For instance, $H_\gamma > H_1$ in the UV mode but $H_\gamma << H_1$ in the EC mode.

We previously attributed these less hydrophobic species to chemically modified sub-isoforms, probably containing very few remaining SH groups verified by the weak electrochemical responses.

For RL-1 prepared at acidic pH, the results tend to be similar, except for having relatively more of the α, β and above all of the γ and ω forms. The MT is probably less "stable" at acidic than at neutral pH: in acidic solution (pH <3), the cations are totally decomplexed and the thiolates become thiols.

On raising the temperature of the column, in 5°C steps in order to carry out sub-isoform separations at different temperatures (25, 30, 35, 40, 50, 55, and 60°C), two main effects can be seen: a logical decrease in retention times of all peaks and a progressive alteration of the α-ω peaks as can be seen in Figure 1. This shows several examples of the elution of the RL-1 sample prepared at neutral pH carried out at various temperatures.

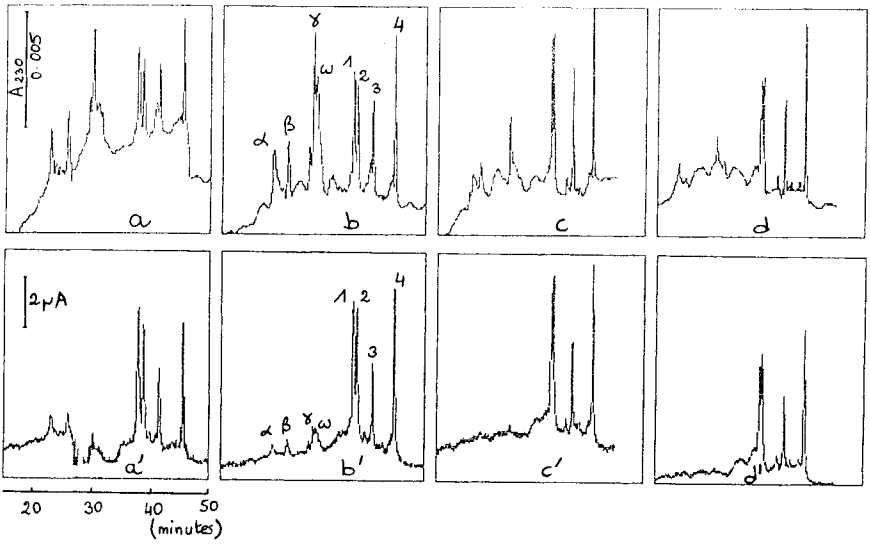

Figure 1. Reverse phase chromatograms of the RL-1 metallothionein at various temperatures for samples prepared at neutral pH. UV detection at 230 nm (0.005 AUFS) and EC detection at 0.6 V. Elution: linear gradient 10-25 % B in 50 min (A = 0.1% TFA, B = 0.1% TFA in acetonitrile) at 1 $mL.min^{-1}$. a, b, c, d: UV detection; a', b', c', d': EC detection; a-a': 25°C; b-b': 35°C; c-c': 55°C; d-d': 60°C.

Retention times: when the temperature is increased from 25 to 60°C, the pressure in the system decreases from 105 to 48 bars consequently due to the decreasing viscosity and, therefore, the retention times become shorter. For instance, the t_R of peak 4 varies from 47.5 to 40.9 min at pH = 3. All peaks follow the same tracks. The initial pH of the sample preparation has no influence on this evolution.

Resolution: when we have a look at the sequence of pictures shown in Figure 1, we can already see qualitatively a decrease in peak resolution with increasing temperature. Let us first consider peaks 1 and 2. At 25°C, R_{1-2} is very good at both pH values and for both detection modes: R_{1-2} = 1.2 (pH = 7, UV), 1.1 (pH = 7, EC), 1.8 (pH = 3, UV) and 1.6 (pH = 3, EC). Thereafter, when the temperature is increased, R_{1-2} reaches a maximum at 30 °C and then decreases sharply: at 60°C, R_{1-2} is < 0.6 in the EC mode (Figure 2). For a given pH, R_{1-2} is always slightly higher in the UV mode than in the EC mode

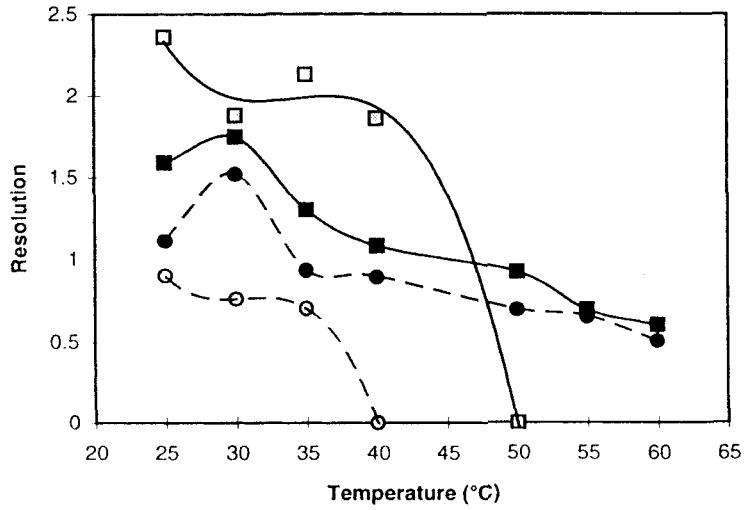

Figure 2. Examples of variations of the resolution of various peaks of RL-1 with temperature; black bullets: $R_{1\text{-}2}$ with EC detection ; grey bullets: $R_{\gamma\text{-}\omega}$ with UV detection; spots: samples prepared at neutral pH; squares: samples prepared at acidic pH.

(about 0.1 difference), but at a given temperature and for both detection modes, the resolution is higher at sample pH = 3 than at 7. At 60°C, the four original forms 1, 2, 3 and 4 are still quite well separated, which is not the case for the other peaks a to w.

At 25°C in acidic medium, the two peaks γ and ω for instance are well separated and $R\gamma_{\text{-}\omega}$ = 2.36. Up to 40°C, this value remains rather constant around $R_{g\text{-}w}$ = 2 (Figure 2). Above 40°C, these peaks γ and ω fuse together and become very small in the UV mode while they disappear completely in the EC mode.

At neutral pH, the resolution $R_{\gamma\text{-}\omega}$ is always much lower than at acidic pH. The γ and ω peaks tend to remain significant in the UV mode until a higher temperature (50°C) than what happens at pH 3, but they start to join together at 35°C (Figure 1). At that temperature, in the EC mode, it is impossible to distinguish γ from ω. Height of the peaks for the main peaks 1, 2, 3, and 4 have to be considered separately from the others.

• Peaks 1, 2, 3, and 4: altogether, the peak heights (or areas) are always higher when the metallothionein is prepared at neutral rather than at acidic pH, for both UV and EC detection modes. When the temperature is raised up, moderate augmentations of the peak intensities can be observed up to 50-55°C before they slightly decrease.

• Peaks α, β, γ, and ω: these peaks behave very differently from the main sub-isoforms. γ and ω are the dominant peaks of this group. In both detection modes, the γ peak goes clearly through a maximum of intensity between 30 and 40°C, before decreasing quickly. The phenomenon is more pronounced at acidic than at neutral pH. It is important to note that α, β, γ, and ω EC signals are very small and always minor compared to those of the original forms, even at lower temperature (25-35°C). Then, from about 50°C at pH = 3 and 55°C at pH = 7, these peaks become insignificant and at 60°C only peaks 1 to 4 are properly separated and detected. The disappearance of the firstly eluted species is slower at neutral pH. Since γ and ω start to merge above a certain temperature, it may be more informative to consider the totality of these two peaks. Obviously, the sum $H_\gamma + H_\omega$ as well as that of areas $A_\gamma + A_\omega$ follow the same variations versus temperature, passing through maximums at 30 - 35°C. Actually, all the peaks α to γ are at their maximum between 30 and 40°C, both in absolute and relative values as shown in Figure 3, where the UV variation of the ratio $(H_\gamma + H_\omega)/(H_1 + H_2)$ as a function of the temperature is presented. This variation is much wider in acidic pH. Above 50°C, the α to γ species do not exist anymore in acidic medium.

Effect of Temperature on RL-2 Sub-Isoform Separation

As we know from our previous works, RL-2 exhibits a much "simpler" polymorphism than RL-1, with one highly dominant sub-isoform (peak 3, $t_R \approx$ 42 min) and some minor peaks (1, 2, and 4, 38.5 min $< t_R <$ 46.5 min), as shown in Figure 4 for a separation carried out at 25°C of a sample prepared at acidic pH. At shortest t_R (≈ 30 min), a rather significant peak is observed in the UV mode but not in the EC mode. These two characteristics (t_R and variable detectability) make this peak similar to the γ peak of RL-1.

The picture is not different at neutral pH. The increase in temperature naturally produces a decrease of all retention times: for instance, between 25 and 60°C, t_R of peak 3 goes from 42 to 37.2 minutes.

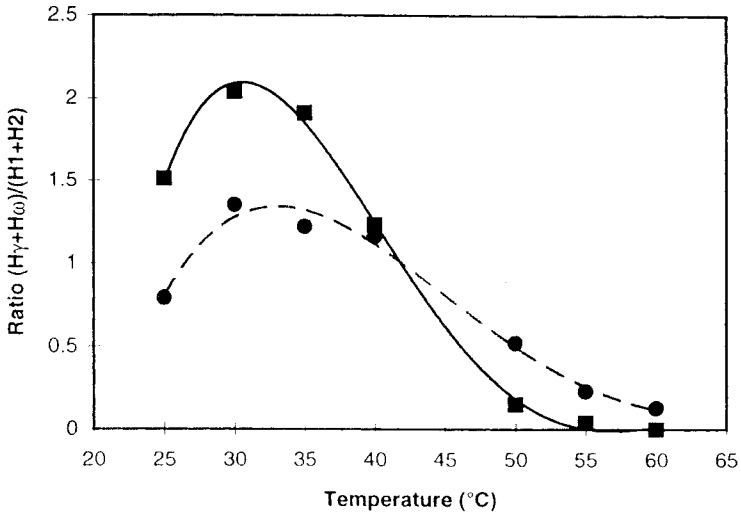

Figure 3. Variation of the UV height ratio $(H_\gamma+H_\omega)/(H_1+H_2)$ of RL-1 with temperature (spots: neutral pH, squares: acid pH).

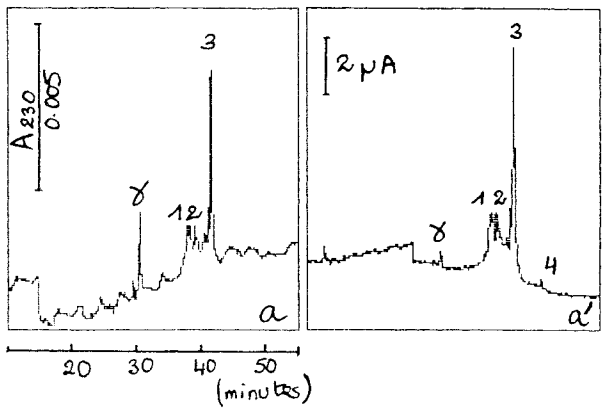

Figure 4. Reverse phase chromatograms of the RL-2 metallothionein at 25 °C, prepared at acidic pH. All other conditions as in Figure 1. a: UV detection; a': EC detection.

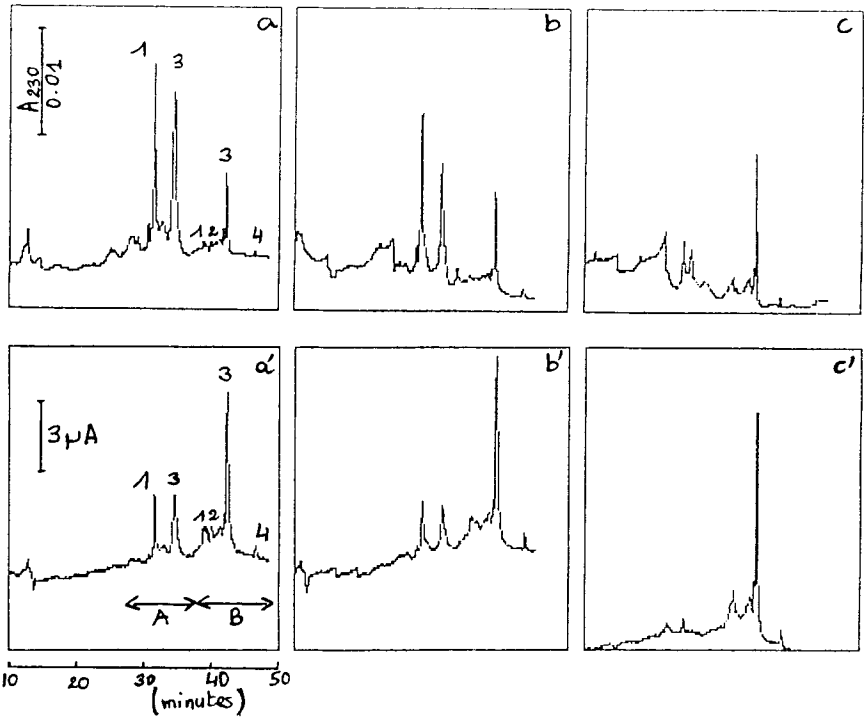

Figure 5. Reverse phase chromatograms of the RL metallothionein at various temperatures, prepared at neutral pH. All other conditions as in Figure 1. a, b, c: UV detection; a', b', c': EC detection; a-a': 25°C; b-b': 40°C; c-c': 60°C.

Concerning peak height, that of the main sub-isoform tends to increase with the temperature, at both pH values and for both detection modes (multiplied by 2 or 3). On the contrary, the γ peak decreases and almost disappears above 50-55°C. At a given temperature, there is no major influence of the preparation pH on the peak intensity of the main peak.

Effect of Temperature on RL Sub-Isoform Separation

The original separations of RL sub-isoforms at both neutral and acid pH conform to those expected at 25°C,[15] with the two groups of peaks A and B (Figure 5): three peaks A, 1A, 2A, and 3A, 2A being very small, followed by four peaks B, of which 3B is largely dominant. Altogether, 1A and 3A are

much bigger than 3B in the UV mode, while in the EC mode the contrary prevails. Group A peaks give relatively low electrochemical responses. The present work, as well as the previous studies, show that peaks 1B, 2B, and 4B correspond to the RL-1 sub-isoforms, while 3B comes from RL-2. Group B peaks correspond to the thiol containing sub-isoforms of the MT-RL. The A peaks are probably due to chemically modified forms, containing few reduced thiol groups.

The increase of temperature will provoke a general decrease of all retention times and a progressive alteration of the A species (Figure 5).

Retention times: they decrease regularly versus the temperature in a similar manner to those observed for RL-1 and RL-2. For instance, between 25 and 60°C, the t_R of peak 1A decreases from 32 to 24.6 min at pH = 7 and from 34 to 26.5 at pH = 3, while the corresponding values for peak 3B are from 42.9 to 35.5 min and from 44.9 to 37.6 min respectively.

Shape of chromatograms and peak height: qualitatively, the effect of increasing tempe-rature on the sub-isoform separation is mostly characterised by a degradation of the group A peaks at higher temperatures. Hence, at 25°C, both H_{1A} and H_{3A} were higher than H_{3B} at both pH values in the UV mode, while at 60°C they were much lower (H_{1A} and $H_{3A} << H_{3B}$ at 60°C). Let us then examine the variation of the peak intensities versus temperature, looking at 1A, 3A and 3B.

- Peak 3B: in both UV and EC modes, we observe an increase of the peak height at both pH values. In fact, there is a real increase until the temperature reaches about 40°C in acidic pH and 50°C in neutral pH, then the height stabilizes thereafter. Altogether, the augmentation is proportionally more notable at acidic pH (H_{3B} is multiplied by 4 between 25 and 60°C) than at neutral pH (H_{3B} has only doubled). But, for each detection mode and at a given temperature, H_{3B} at pH = 7 > H_{3B} at pH = 3.

- Peak 1A: this peak, as well as 3A, behaves oppositely to 3B. At pH = 3, when the temperature of the column increases, H_{1A} increases first slightly up to 40°C before decreasing sharply, although at 55°C it is still a notable peak. At pH = 7, we observe a direct decrease of the peak height. At both pH values, peak 1A is small at 60°C but remains appreciable in acidic pH when detected by UV. On the whole, whatever the temperature this peak is almost always higher when the MT is prepared in acidic medium than in neutral medium.

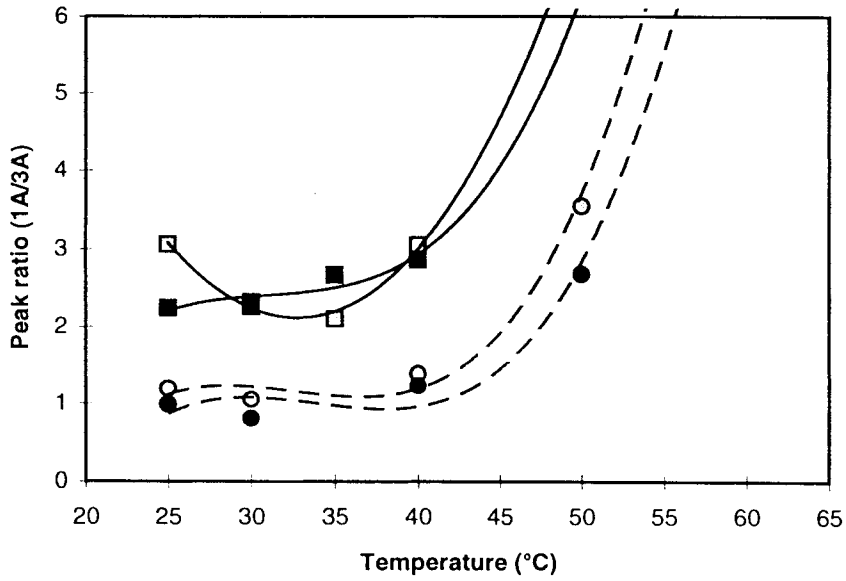

Figure 6. Variation of the peak ratio 1A/3A of RL with temperature (spots: neutral pH, squares: acidic pH). Black bullets: EC detection; grey bullets: UV detection.

• Peak 3A: for this peak, the sample preparation pH seems to play an even bigger role than what we had for the other peaks. At neutral pH, the peak is very significant at 25°C, as high as 1A was, and then it decreases very quickly as soon as the temperature is raised up. In acidic pH, the type of variation of 3A is quite similar to that of 1A with a height maximum at 35°C but followed by a more rapid decrease. Whatever the pH, the peak decreases to zero at 55-60°C. For each temperature, H_{3A} is higher at neutral than at acidic pH.

• Ratio 1A/3A: between 25 and 35 °C, the ratio H_{1A}/H_{3A} shows only very moderate variations, but above 40°C, it tends to increase very strongly (Figure 6). This holds true for both detection modes. At room temperature both peaks are important, but with increasing temperature 3A evolves towards a complete disappearance which is not exactly the case of 1A, hence these two species have some chemical differences. 3A seems to be a particularly heat-unstable form.

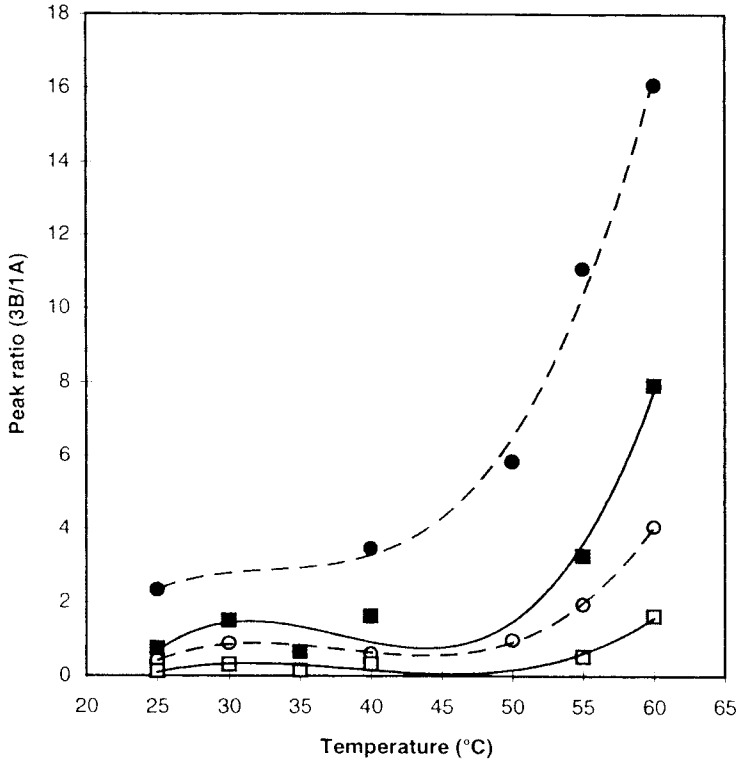

Figure 7. Variation of the peak ratio 3B/1A of RL with temperature (spots: neutral pH, squares: acidic pH). Black bullets: EC detection; grey bullets: UV detection.

- Ratio 3B/1A: this ratio summarizes the coupled behaviour of peaks from groups A and B quite well. Between 25 and about 40°C, it remains more or less stable at both pH values and for both modes of detection (Figure 7). For a given temperature, the ratios are higher at neutral than at acidic pH. From 40°C and up, the ratios increase very quickly and dramatically: between 25 and 60°C, the UV and EC ratios are multiplied about 10 times. The more the temperature increases, the less significant group A peaks become.

From all the experiments carried out on the effects of temperature on MT sub-isoforms separation, it appears that the range of 30-40°C corresponds to a critical domain in which:

a) there are very good resolutions of peaks of RL-1 (examples of $R_{1\text{-}2}$ and $R_{\gamma\text{-}\omega}$);
b) there are maximums of response for peaks γ and ω of RL-1, resulting in maximum ratios $(H_\gamma + H_\omega)/(H_1 + H_2)$;
c) group A peaks of RL are either at their maximum height (acidic pH) or still very high (neutral pH).

Therefore, at an average temperature of 35°C, conditions are attained where the main peaks (those equally detected in both UV and EC modes) are perfectly separated and detected at both preparation pH values, and where the other peaks (not equally detected by UV and EC) are almost at their maximum, providing a situation in which all original and chemically oxidised sub-isoforms are present. For this reason, the following steps in this study were carried out at 35°C.

Mixture of RL-1 + RL-2

Two solutions containing both RL-1 and RL-2 were prepared at neutral and at acidic pH respectively and comprising of 20% RL-1 and 80% RL-2. These proportions were found by us to be more or less those of the original RL.[14-15,21] The elution was then performed at 35°C.

At neutral pH, the chromatogram of RL-1 + RL-2 corresponds very well to the superposition of those of RL-1 and RL-2 with the appropriate coefficients, 0.2 and 0.8 respectively: peak 3 coming from RL-2 is dominant and peaks 1, 2, and 4 are well separated and detected in both UV and EC modes (Figure 8).

Peaks α, β, and ω coming exclusively from RL-1 are of course very small, while γ, found in both RL-1 and RL-2 is significant in the UV mode. In the EC mode, these α-ω are insignificant. The situation is quite similar at acidic pH, except for there being a very high γ peak.

When the chromatogram of the mixture is compared to that of an original RL (1 + 2) solution, we observe a perfect correspondance of peaks 1, 2, 3 and 4 of RL-1 + RL-2, with the group B peaks of RL (1B, 2B, 3B and 4B), at both pH values and for both detection modes. Concerning the other peaks, γ corresponds qualitatively exactly to 1A and ω to 2A, while 3A remains with no "partner" peak in the mixture.

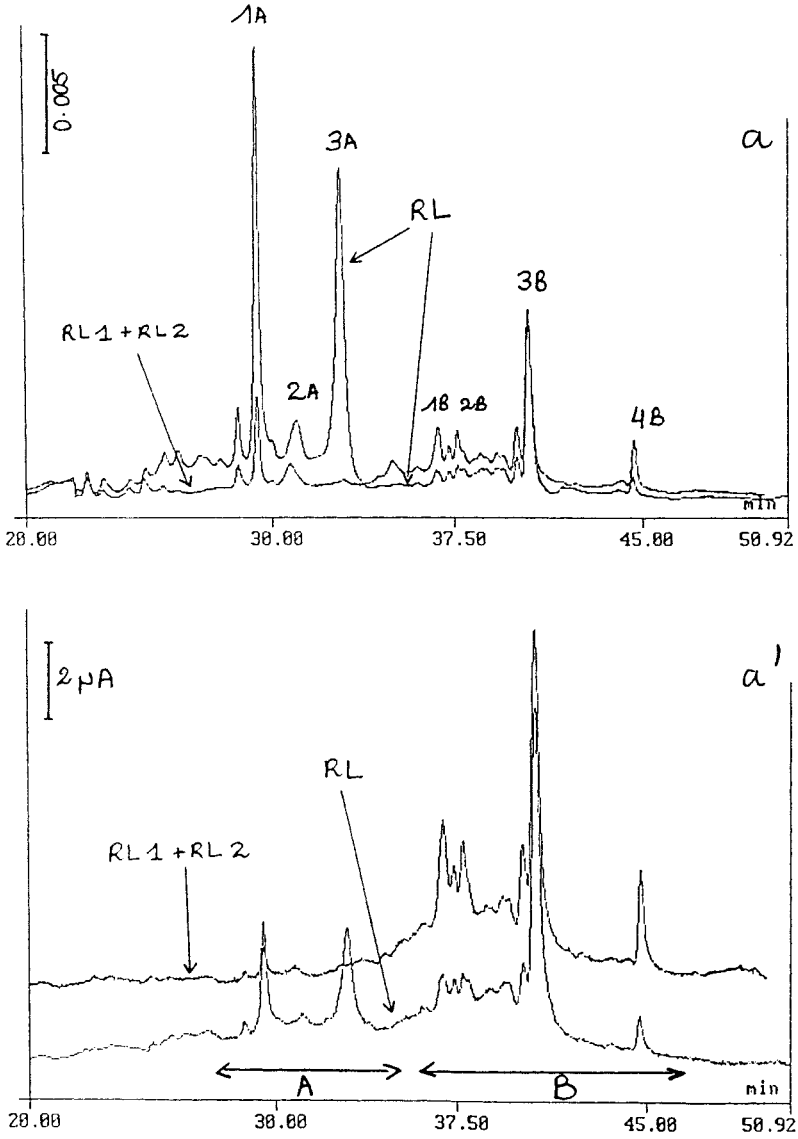

Figure 8. Comparison of the chromatogram of a RL solution with that of a mixture of 20% RL-1 + 80% RL-2 at 35°C, both prepared at neutral pH. All other conditions as in Figure 1. a: UV detection ; a': EC detection.

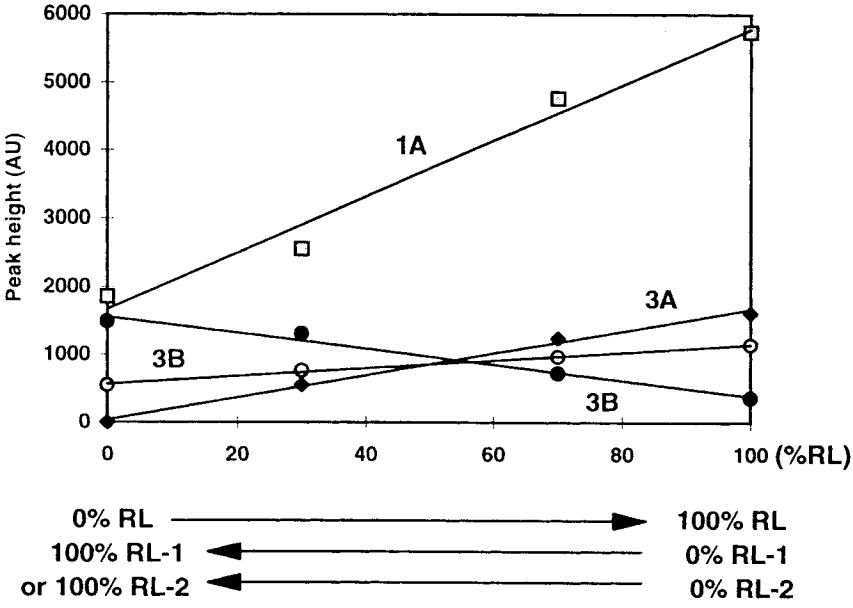

Figure 9. Variation of the UV height of peaks for mixtures RL + RL-1 and RL + RL-2 of various compositions, at 35°C. Chromatograms were obtained under the same conditions as in Figure 1. Black bullets: solutions RL+ RL-2; grey bullets: solutions RL + RL-1; spots: peak 3B; squares: peak 1A; diamonds: peak 3A.

This large peak does not seem to result from a direct contribution from RL-1 nor from RL-2: mixing the two isoforms does not generate peak 3A which is largely found in the original RL. This experiment enhances the previous result concerning the difference in the chemical nature of 1A and 3A.

Mixtures of RL + RL-1 and RL + RL-2

Several mixtures RL + RL-1 and RL + RL-2 have been prepared at both pH values and eluted at 35°C, in order to confirm the assignment of the various peaks. In Figure 9 the UV response variations of the three dominant peaks of RL (1A, 3A, and 3B) as a function of the sample composition are shown. From previous experiments, we assume that 1A of RL is equivalent to γ of RL-1, that 3A has no equivalent in either RL-1 or RL-2, and that 3B originates from peak 3 of RL-2 (and RL-1). Let us consider these RL peaks successively.

1A: the identity of 1A of RL with that of γ of RL-1 has been tested with RL + RL-1 solutions: we can see from Figure 9 that the signal of this peak is much bigger in pure RL than in pure RL-1 and that it covaries nicely with the proportions of RL and RL-1, decreasing with increasing proportions of RL-1 (H_{1A} = 41.2 %RL + 1671, r = 0.980).

3A: when RL-1 or RL-2 (case presented in Figure 9) is added in increasing proportions to a solution of RL, there is an obvious decrease of peak 3A; as it is only found in RL, its height follows the proportion of RL perfectly with an intercept of the equation H = f (%RL) close to zero (H_{3A} = 16.4 %RL + 36, r = 0.995 for a mixture RL + RL-2).

3B: this peak corresponds to peak 3 of RL-1 and of RL-2; it is the dominant species in RL-2 and its intensity logically decreases when the proportion of RL-2 decreases (H_{3B} = -11.8 %RL + 1561, r = 0.980). On the contrary, peak 3 is a minor peak in RL-1 (probably a remaining contamination of RL-2) and therefore its signal slightly increases when the proportion of RL-1 decreases (H_{3B} = 5.9 %RL + 562, r = 0.996).

DISCUSSION

This work confirms the fact that when submitted to a gradient elution in an acidic medium (TFA) on a reverse phase column and at room temperature, the metallothioneins extracted from rabbit liver RL (1 + 2), RL-1, and RL-2 exhibit two types of peaks: firstly, those equally detected by UV and coulometric detection and attributed to the original putative thiol containing sub-isoforms of the MTs (or more exactly aposub-isoforms since the metals are decomplexed due to the acidic pH of the mobile phase) and secondly those eluted earlier, detected by UV but hardly at all by EC and attributed to oxidised forms, containing very few remaining thiol groups but having disulphides bonds instead and, therefore, not being detectable in the EC mode. With this work, we have reinforced the attribution of the various peaks.

Obviously, the sample pH plays an important role on the absolute and relative intensities of the various peaks. The thiolic sub-isoforms (peaks 1-4 of RL-1 and RL-2, group B peaks of RL) always give bigger peaks when the MTs are prepared in neutral solution. On the contrary, the supposed oxidised forms (α-ω of RL-1, group A peaks of RL except 3A) give higher responses when the MTs are prepared in acidic medium. Therefore it seems that the acidic pH enhances the formation of the oxidised species from the original reduced forms. Peak 3A does not follow this rule which again underlines its different nature and unexplained origin for the moment.

Moreover, the hydrophobic nature of the stationary phase and the low pH and organic content of the mobile phase are all factors known to generally induce conformational alterations of proteins in a reverse phase column. It would, therefore, be reasonable to think that these conditions, which is the case for us, could at least partly explain our observations. In simpler words, the oxidised species would be formed in the column due to the experimental conditions. However, this cannot account for everything as we have already shown that there are natural evolutions with time of MT solutions kept at room temperature moving in the direction of the formation of less hydrophobic species giving UV responses but very low electrochemical signals.[15] As an illustration, we can look at the nice case of an RL-2 sample prepared at pH = 3, eluted immediately (t = 0), kept at room temperature, and re-eluted 18 days later under, of course, the very same conditions (Figure 10). On the original chromatogram, we see the classical picture of RL-2, one dominant sub-isoform detected in both UV and EC modes. After the 18 days, this main peak has strongly regressed and a new species has been formed, eluted earlier, well detected by UV, but not at all by EC. Clearly this form is not due to the chromatographic experimental conditions.

The present study reveals that the variation of temperature also appears to be an important parameter for the discrimination between the two types of species. Starting at around 50°C, we observe a drastic alteration of the first peaks of RL-1 (α-ω peaks) and of RL (group A peaks) with their quasi disappearence at 60°C, at both sample preparation pH, acid and neutral. At the same time, the images of the main thiolic sub-isoforms remain qualitatively rather unchanged, but they display peak intensity variations which generally increase with increasing temperature. At 60°C, the electrochemical detection shows a situation where only original thiolic sub-isoforms are seen. Remembering that one of the typical properties of MT is their heat-stability, this work, therefore, demonstrates that the first eluted species could hardly be attributed to MT sub-isoforms originally present.

To summarise, in the temperature range 20-40°C with optimum values at 35°C, all peaks are present, original reduced, and then oxidised forms, well resolved and showing excellent responses in both UV and EC modes, while at 60°C, only putative original sub-isoforms are observed.

Besides these important results, the temperature variation has another evident effect, the change in retention time of the various peaks. More generally, it is known that in HPLC temperature has a significant influence on the column back pressure, since it is proportional to the viscosity of the mobile phase.

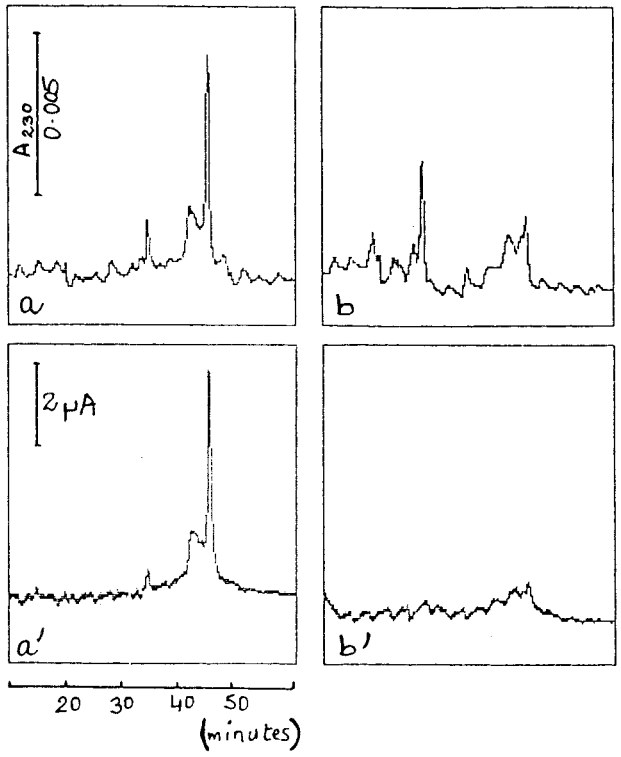

Figure 10. Reverse phase chromatogram of a RL-2 solution prepared at acidic pH, eluted immediately at t = 0, kept at room temperature and re-eluted 18 days later. All other conditions as in Figure 1. a-b: UV detection; a'-b': EC detection; a-a': t = 0 days; b-b': t = 18 days.

In our case, the pressure dropped from 105 bars at 25°C to 48 bars at 60°C, therefore increasing the diffusion rates and consequently the mass-transfer rates between the mobile phase and the solid phase.[22-24] Thus, the effect of temperature change on the retention is, for most chromatographic separations, essentially due to the enthalpy change of the solute-solid phase interaction. The solute retention is related to the thermodynamic equilibrium partition coefficient K, following the equation $k' = K.\Phi$ where k' is the capacity factor and Φ the phase ratio of the column which is a constant for a given column and mobile phase. The variation of free energy in the interaction process is given by :

$$\Delta G° = \Delta H° - T.\Delta S° = - R.T.\ln K = - R.T.\ln k'/\Phi$$

from which we have

$$\ln k' = -\Delta H°/R.T + \Delta S°/R + \ln \Phi$$

where $\Delta G°$, $\Delta H°$ and $\Delta S°$ are the changes of standard free energy, of enthalpy and of entropy of the retention reaction respectively, R is the molar gas constant and T the absolute temperature. This is the fundamental relationship between temperature and chromatographic retention (Van't Hoff plot). It has already been shown almost twenty years ago that plotting ln k' versus 1/T leads, in most cases, to linearity,[25,26] due to an enthalpy-entropy compensation.[27] From the slope $-\Delta H°/R$, the variation of enthalpy can be obtained directly. For a temperature range of 5 to 100°C, an enthalpy change of about -5 kcal.mol^{-1} appears to be a typical value in RP-HPLC.[28] Stronger variations can occur, depending on the size of the molecule. For small molecules, the enthalpy changes were found to range from -8 to -5 kcal.mol^{-1} for temperature varying between 20 and 50°C.[29] Chen and Horváth[23] calculated retention enthalpies averaging at about -4 kcal.mol^{-1} for nitrobenzene between 40 and 120°C. On the contrary, enthalpy changes are expected to be much larger for bigger molecules such as peptides, proteins and other macromolecules. For instance, for lysozyme (MW = 14400 Da), they ranged from -38 to -12 kcal.mol^{-1} depending on the mobile phase conditions used (TFA/various proportions of acetonitrile).[23] However, a deeper survey of different studies carried out on this topic often shows much lower enthalpy variations for peptides and proteins than those referred to above.[23,28] For example, cyanogen bromide fragments of hemoglobin, for which Mahoney and Hermodson[17] observed a decrease of retention times between 29 and 50°C (0.15 min/°C) corresponding to enthalpy variations of -1 to -2 kcal.mol^{-1} (TFA/acetonitrile system). The same order of values has been published by Guo et al.[20] for five synthetic decapeptide standards between 26 and 66°C ($\Delta H°$ = -1 to -5 kcal.mol^{-1} in TFA/acetonitrile). We can also mention the work of Ingraham et al.[19] on several proteins: between 0 and 47°C, they noticed decreases in retention times for cytochrome C and myoglobin for which we calculated $\Delta H°$ lower than -1.5 kcal.mol^{-1} (TFA/acetonitrile).

We then applied the Van't Hoff plot to our own results, establishing the ln k' = f(1/T) relationship for all peaks of the three MTs. For all of them, a very good linearity is observed with correlation coefficients ranging from 0.919 to 0.999. The slopes of these linear curves allow one to calculate their respective enthalpy variations which are given in Table 1. All values range between about -1 and -2 kcal.mol^{-1}. There is also a slight trend of a given MT having larger

Table 1

Van't Hoff Plot for the Main MT Peaks*

MT	Peak	ln k' = f(1/T)	r	$-\Delta H°$ (kcal.mol^{-1})
	α	1006.5/T - 1.110	0.9445	2
	γ	791.7/T - 0.137	0.9586	1.57
RL-1	1	592.8/T + 0.732	0.9745	1.18
	3	476.1/T + 1.205	0.9876	0.95
	4	480.3/T + 1.288	0.9955	0.96
RL-2	3	461.9/T + 1.252	0.9190	0.92
	1A	715.7/T + 0.086	0.9405	1.42
RL	3A	794.1/T - 0.089	0.9302	1.58
	3B	515.8/T + 1.063	0.9612	1.03

* Enthalpy changes associated with temperature variations (25-60°C).

k' associated with lower $\Delta H°$. This means that there is larger heat of transfer for the less retained species. On the whole, the effect of temperature on the MT sub-isoform retention times is moderate. We fit the case close to that described by Guo et al.,[20] showing that enthalpy changes associated to proteins are not always high.

In conclusion, it appears that the variation of temperature on MT sub-isoform separation by RP-HPLC in a TFA/acetonitrile gradient has two main effects. A moderate one on the heat of transfer of each sub-isoform and, therefore, on their respective retention times, but a very significant one on the type of species detected: at high temperature (60°C) only putative original thiol containing forms are observed while at lower values (25-40°C), disulphide containing oxidised forms are also present. This work shows the high value of using an on-line UV-coulometric detection system for this type of molecule.

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