Table 1 (abstract P16) Specific activity (in U/ng) of -galactosidase and its derivative VP1LAC produced in different strains, in the soluble and insoluble fractions.

Strain	Solublefraction	Insolublefraction
MC4100/pJCO46	628.2 ± 40.5	6.3 ± 0,3
MC4100/pJVP1LAC	234.1 ± 52.9	65.2 ± 19,4
BB4565/pJCO46	689.7 ± 164.9	63.6 ± 2,2
BB4565/pJVP1LAC	230.2 ± 25.7	129.6 ± 45,9
JGT20/pJCO46	888.9 ± 179.3	175.2 ± 34,9
JGT20/pJVP1LAC	12.5 ± 3.8	10.3 ± 6.3

contrary, protein deposition as inclusion bodies renders homogeneous but strongly evolving structures. In this context, the specific activity of enzyme-based inclusion bodies is much higher than in the equivalent thermal aggregates, by a mechanism that might be controlled by the chaperone DnaK. Protein deposition as inclusion bodies is then a cell driven complex process through which misfolded protein forms but also functionally competent polypeptides are efficiently packaged.

Acknowledgements
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P17

Point mutation of serine 179 in the human Prolactin (PRL) affects recombinant protein expression, folding and secretion, abolishes PRL nickel (II)-binding and increases heparin binding capacities

Eric Ueda[1], Carlos Soares[1], Ameae Walker[2] and Paolo Bartolini[1]
[1]Biotechnology Department, IPEN-CNEN, Cidade Universitaria, Sao Paulo, Brazil
[2]Division of Biomedical Sciences, The University of California, Riverside, CA, 92521, USA

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Background: S179D prolactin (S179D PRL) is a pseudophosphorylated form of human prolactin (PRL) which has inhibitory effects on tumor growth [1] and angiogenesis [2]. The S179D PRL preparations used for these experiments consisted of properly refolded inclusion bodies (IB) from *Escherichia coli* [3]. Trying to attain a better folded mutant, we used secretion expression based systems. However, single point mutations can affect protein periplasmic expression [4], and secretion from mammalian cells [5]. We observed that upon a mutation of Serine 179 to an Aspartate, expression was nearly abolished when compared with PRL in *E. coli* periplasm, while the cytoplasmic product was more prone to proteolysis. Using eukaryotic cells we were able to produce preparations comparable to IBs in terms of bioactivity. We also demonstrated that this mutant had a higher affinity for heparin and lower binding capacity towards divalent metals (M (II)).

Results: S179D PRL periplasmic expression was very low when compared to PRL. Use of different promoters, different signal peptides or different activation temperatures had no effect (Figure 1).

MALDI-TOF spectrometry was carried out for identity of S179D PRL in the extracts (Figure 2).

BL21 strain was used (Figure 1B) without improvements for S179D PRL expression (Table 2).

We used BL 21 codon plus® in order to investigate the GC-, AT-rich sequence of the PRLs influence on expression. This strain did not rescue expression of S179D PRL or PRL (Figure 1C). pTac induction at lower temperatures should encourage protein solubility and folding in the cytoplasm [6]. We carried out cytoplasmic expression with an Origami B strain, in which cytoplasm folding is facilitated [7]. Surprisingly, when S179D PRL was produced in soluble form, unlike PRL, low molecular forms were observed (Figure 3A and 3B), and also in BL21, cleaved forms and soluble high molecular aggregates were present (Figure 3A). pL constructs had very low yields for both PRLs (Figure 3).

An eukaryotic expression system was chosen to successfully produce soluble, monomeric, recombinant S179D PRL.

B-casein bioassays were carried out to check S179D PRL folding. (Figure 5).

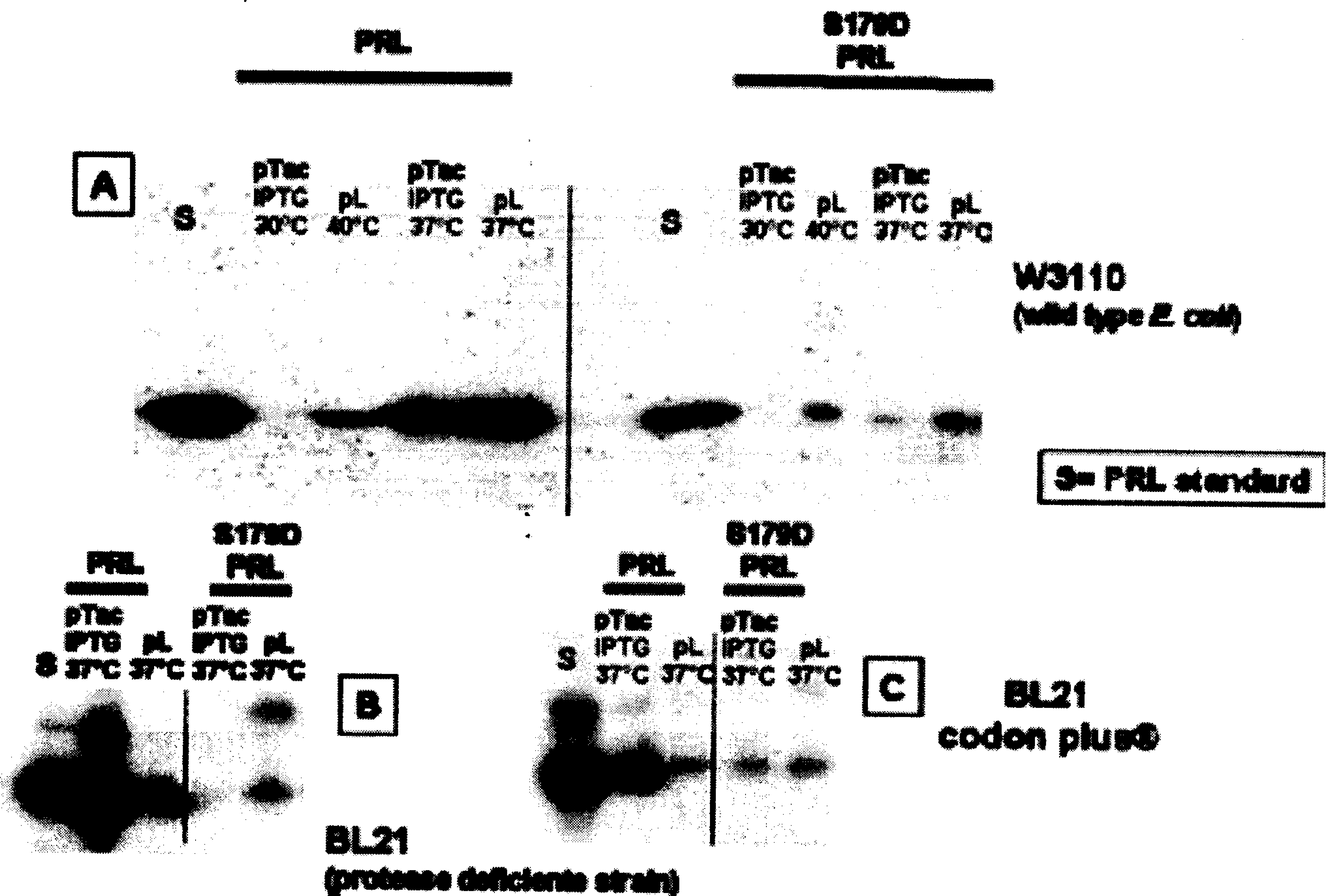
Moreover S179D PRL had a decreased affinity towards Ni (II) ans Zn (II). On the other hand it had an increased affinity towards heparin.

Table 1 (abstract P17) Protein expression yield (g/mL/OD) and final optical densities (OD_{600}) of different strains with pL promoter.

	E. colistrain	Protein yield (g/mL/OD)	Final OD_{600}
PRL	W3110	1.3 ± 0.2	4.0 ± 0.3
	BL21	1.9 ± 0.4	1.3 ± 0.2
	BL21 codon plus	1.4 ± 0.3	1.0 ± 0.2
S179D PRL	W3110	0.34 ± 0.03	3.8 ± 0.6
	BL21	0.35 ± 0.5	1.3 ± 0.5
	BL21 codon plus	0.40 ± 0.3	1.2 ± 0.1

11490

Figure 1 (abstract P17)



A, B and C, Immunoblots of periplasmic extracts.

Conclusion: We tried to produce a correctly folded form of S179D PRL, already obtained as refolded IBs [3]. Unexpectedly, this point mutation of PRL impaired protein expression, and was not related to the strain, protease degradation of our protein, or preferential codon usage (Figure 1). To avoid proteolysis and misfolding we used lower temperatures during protein production [8], but it failed to produce S179D PRL. Low levels of S179D PRL were only detected by immunoblots (Figure 1) and by immunoassay (table 1). Expression of soluble S179D PRL in the cytoplasm of E.coli was not efficient either, as denoted by soluble aggregates and cleaved S179D PRLs. Eukaryotic expression systems have a better folding machinery, being difficult-to-fold proteins more easily expressed [9]. Thus, we successfully produced S179D PRL at RP-HPLC detectable levels (Figure 4). MALDI-TOF analysis showed that all samples had the expected molecular weight (Figure 2). RP-HPLC demonstrated that S179D PRL had a different folding than PRL. The bioactivity assay showed that all preparations of S179D PRL were correctly folded. S179D PRL also showed physical-chemical differences, having a lower M (II)-affinity and a higher heparin-affinity. This confirms reports of PRL mutants with low Zn (II) affinity that are poorly secreted [4] and also could account for its anti-angiogenic effect [2, 10].

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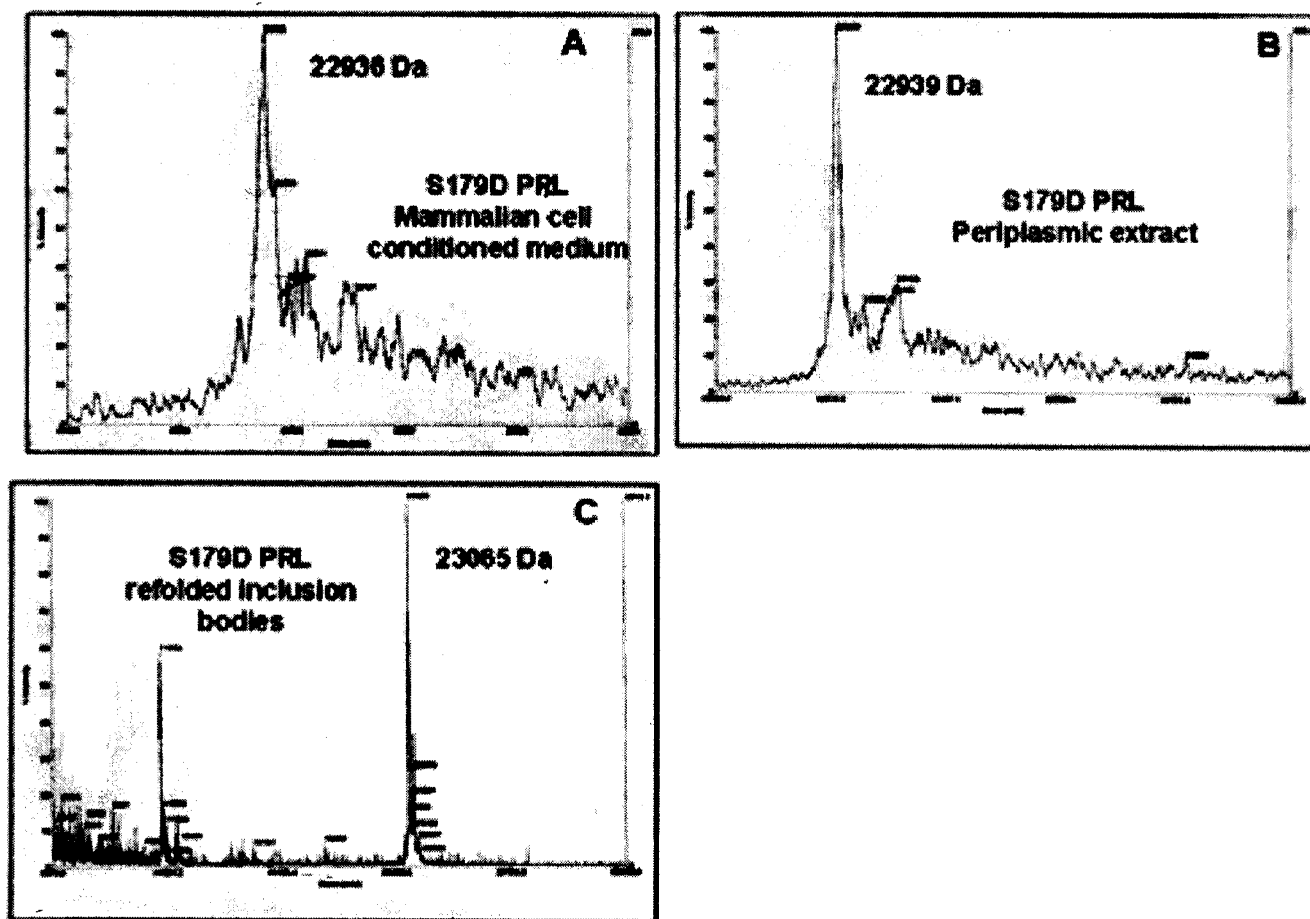
Figure 2 (abstract P17)



Molecular masses determined by MALDI-TOF-MS.

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P18

Models for the study of inclusion bodies formation as a function of fermentation conditions and protein sequence

Pietro Gatti-Lafranconi[1], Diletta Ami[1], Antonino Natalello[1], Gaetano Invernizzi[1], Ario de Marco[2], Silvia Maria Doglia[1] and Marina Lotti[1]
[1]*Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, 20126 Milano, Italy*
[2]*EMBL Scientific Core Facilities, Mayerhofstrasse 1, D-69117, Heidelberg, Germany*

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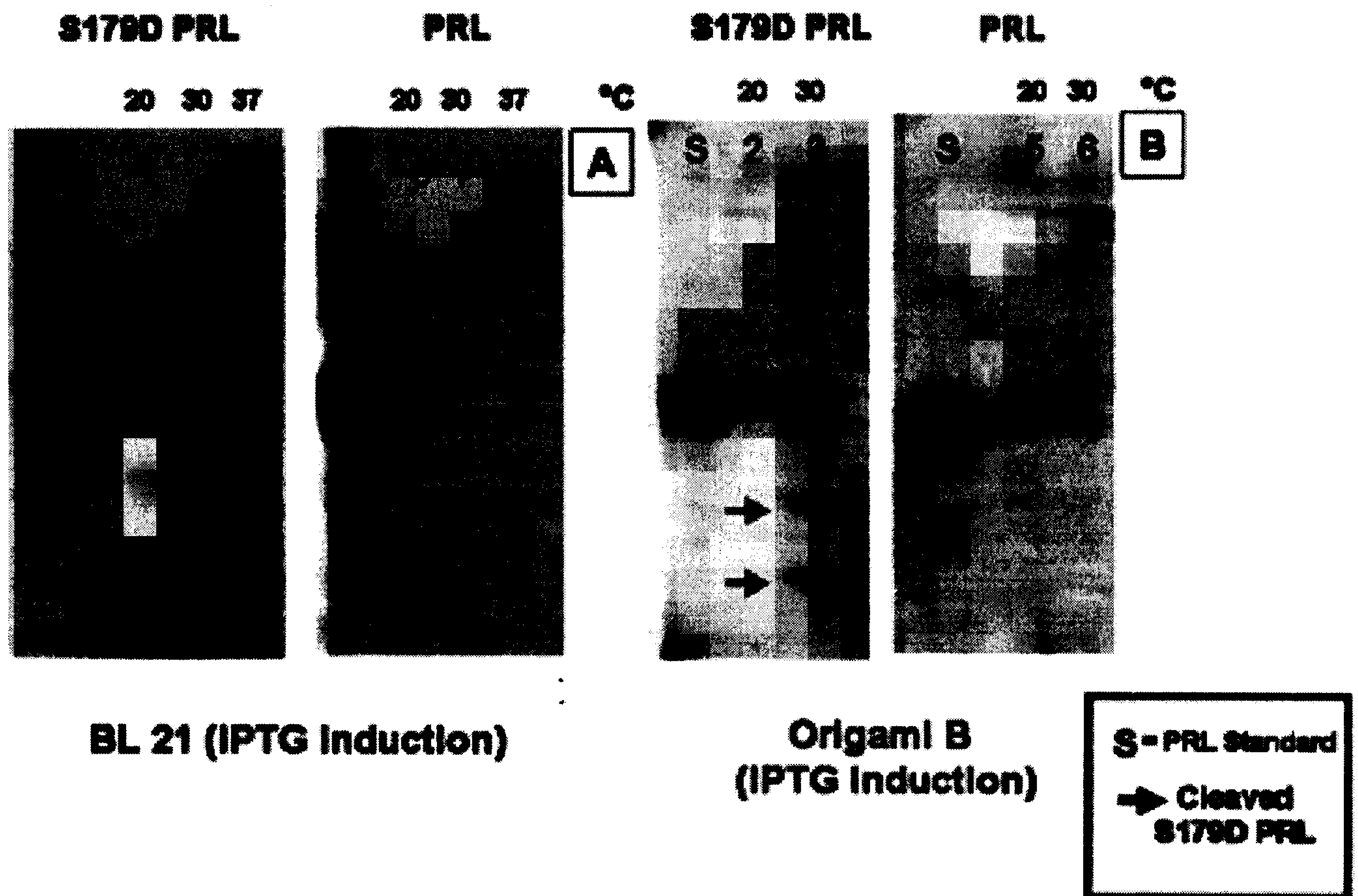
Background: The building of aggregates of variable complexity is often observed in bacterial host cells upon over-expression of recombinant proteins. This event is thoroughly studied as it impacts on the production of recombinant proteins and also as a model to investigate the molecular and physiological factors producing protein aggregation in living cells [1]. In *E. coli*, a network of molecular chaperones assists protein folding and re-folding [2]. Recent experiments showed that aggregation reversion can be improved as protein synthesis is interrupted and that the ratio protein/

Figure 3 (abstract P17)



Immunoblots of soluble fractions of *E. coli* lysates.

chaperons as well as the kind of chaperons enclosed in inclusion bodies varies according to the physiology of overproduction [1, 3, 4]. Stability, solubility and propensity of a protein to aggregate both in inclusion bodies and in amyloid structures have been related to its polypeptide sequence [5]. This study aims at gaining a deeper insight in the composition and kinetics of aggregate formation and to relate this information to the molecular features of the recombinant expressed proteins.

Results: We have studied the behaviour of three different proteins over-expressed in *E. coli* by commercial expression vectors regulated by IPTG. The three model polypeptides display different features: (i) the cold active lipase from *Pseudomonas fragi* is very unstable even at moderate temperature and, therefore, is a very sensitive tool to investigate the temperature-dependent aggregation development [6]; (ii) the green fluorescent protein-glutatione S- transferase fusion protein enables to monitor residual fluorescence in aggregated proteins and, as a consequence, to evaluate the extent of residual native structure; (iii) wild type and mutated lactoglobulins have been used as a probe to test the effect of changes in the amino acid sequence on the protein stability during recombinant expression. Ratio of soluble to insoluble proteins has been evaluated by SDS PAGE and activity has been measured in both fractions, whenever applicable. Additional information has been provided by structural analysis performed by Fourier transform infrared spectroscopy. In all the models the fermentation temperature has been identified as a major determinant of the total amount and rate of aggregation as well as of the complexity, compactness and residual native- like structure of inclusion bodies [4, 5, 6, 7]. Moreover, concentration of DnaK inside inclusion bodies has been followed by Western-blot analysis and a correlation with the amount of insoluble protein has been detected.

Conclusion: Kinetics of aggregation, content and residual protein structure and activity in *E. coli* inclusion bodies are features highly protein specific but also dependent on the conditions under which aggregation occurred. This last observation suggests that the precise monitoring of recombinant protein aggregation during the fermentation can lead to a system in which the optimisation of the growth conditions is automatically set with the biotechnologically relevant increase of soluble protein yields. Furthermore, the monitoring of the aggregation dynamics specific for the different mutants might provide valuable indications to engineer species with higher solubility.

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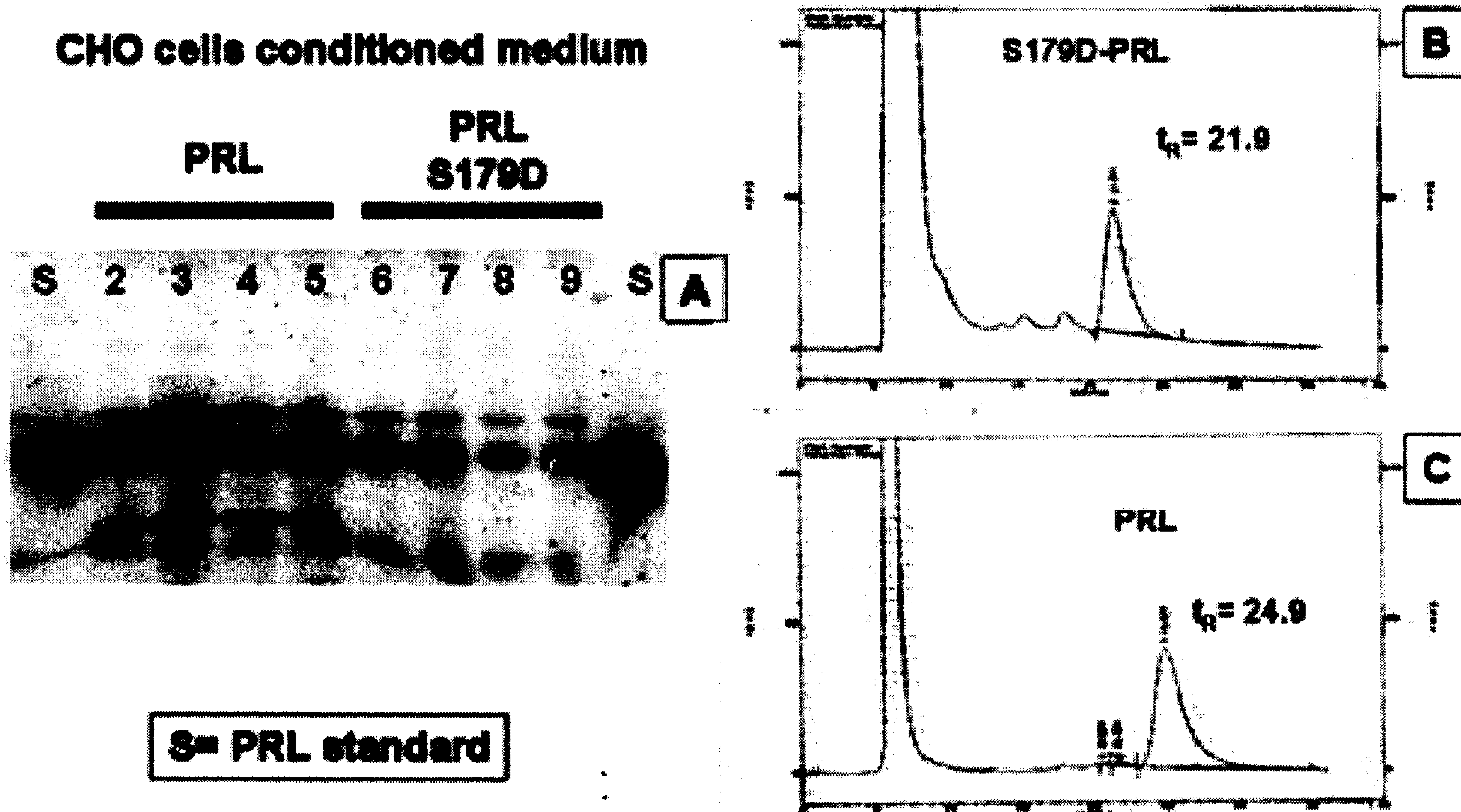
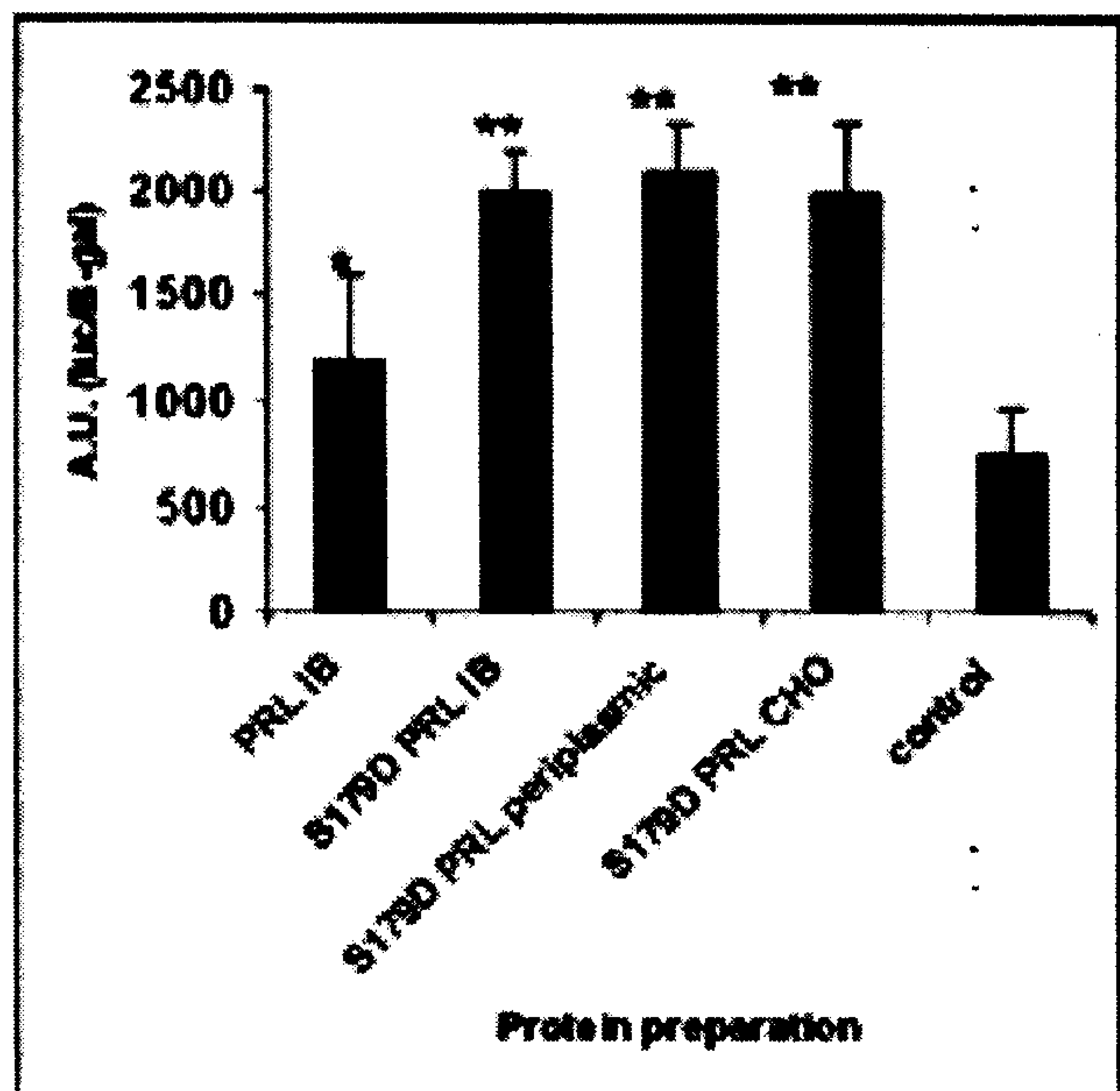
Figure 4 (abstract P17)



Immunoblot of conditioned medium. **B, C,** RP-HPLC analysis.

Figure 5 (abstract P17)



-casein bioassay. * $p < 0.05$ versus control; ** $p < 0.01$ versus control. AU, arbitrary unit.

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P19

Addition of Repressor in inducible promoter system improves soluble expression of recombinant protein in *E. coli*

Kyung-Hwan Jung
Department of Food and Biotechnology, Chungju National University, Chungju 380-702, Chungbuk, Korea