Acid protease purification by dye affinity chromatography

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Purificación de las proteasas ácidas mediante cromatografía de afinidad de los colorantes.

Purificació de proteasa àcida per cromatografia d'afinitat de colorants.

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RESUMEN

Luego de ensayar 9 colorantes triazínicos inmovilizados, el Rojo F5B fue seleccionado como el mejor ligando para la purificación de la quimosina y la proteasa ácida de Mucor miehei por cromatografía de afinidad, a pH 6,5 y 5,4 respectivamente. Para la purificación de la proteasa de Mucor bacilliformis a partir del extracto crudo de un cultivo en sustrato sólido, se seleccionó el Rojo HE-3B, que permitió recuperar 95,2% de enzima pura.

Palabras clave: Proteasa Ácida. Purificación. Cromatografía de Afinidad. Colorantes Triazínicos.

SUMMARY

After screening of 9 immobilised triazinic dyes, Red F5B was selected as the best ligand for chymosin and *Mucor miehei* acid protease purification, at pHs 6.5 and 5.4 respectively. For *Mucor bacilliformis* protease purification from a crude extract of a solid-state culture, Red HE-3B was selected, allowing a 95.2% recovery of pure enzyme.

***Keywords:* Acid Protease. Purification. Affinity Chromatography. Triazinic Dyes.**

RESUM

Per a la purificació de la proteasa àcida, després d'assajar 9 colorants triazínics immobilitzats, el Roig F5B fou el triat com a millor ligand per a la quimosina i Mucor miehei a pH 6,5 i 5,4, respectivament. Per a la purificació de la proteasa àcida del Mucor bacilliformis a partir d'un extracte de cultiu en estat sòlid, fou triat el Roig HE-3B. Que va permetre una recuperació de l'enzim pur del 95,2%.

Mots clau: Proteasa àcida. Cromatografia d'afinitat. Colorants triazínics.

INTRODUCTION

Proteases are an important group of industrial enzymes. Among them, acid proteases such as natural or recombinant bovine chymosin are mainly used in cheese manufacture. In addition, acid proteases from *Mucor miehei* and *Mucor pusillus* are widely utilised as substitutes for calf chymosin. Depending on the source (fungal cultures, heterologous expression or calf stomach), different purification degrees are required before sending the product to the market.
Triazine dyes are used as ligands for affinity chromatography as an alternative technique to ion exchange chromatography. Taking advantage of a protein pseudobiospecific interaction, dye affinity chromatography offers a number of positive features for large-scale processing: low cost and ready availability of the dye, stability against chemical and biological degradation, and easy immobilisation[1].
Chromatography on immobilised dyes is seldom used for protease purification. On the other hand, it is very often used for separation of dehydrogenases or kinases[2]. It is evident from crystallographic data of dye-protein complexes[3] that a precise fit between one dye structure and an acceptor crevice on a protein surface can explain the strong affinity of a dye for one given protein, and hence its selectivity. The dye for a purification procedure is chosen from among the few immobilised dyes that are readily available. However, screening many different immobilised dyes to find one or several of them that interact selectively with the target protein can be rewarding[4].
Immobilised Cibacron Blue and Red HE-3B was used to purify mainly alkaline or neutral proteases. However, within the acid protease family, only chymosin was purified (by dye-affinity chromatography in the positive mode[5]). Chymosin from rennet was retained in 25 mM citrate, pH 5.5, while pepsin passed through. Chymosin was eluted with 1.7 M NaCl in the same buffer or 50% ethylene glycol in 25 mM citrate, pH 6.2.
The aim of this work was to test different triazine dyes and chromatographic conditions that could be useful for affinity purification of chymosin and the acid proteases from *M. miehei* and *M. bacilliformis*.

MATERIALS AND METHODS

Materials

Sepharose 4B and PD-10 columns were from Amersham Pharmacia, Uppsala, Sweden. Triazine dyes and the *M. miehei* acid protease were from Sigma-Aldrich, St. Louis, USA. Bovine chymosin (ChymaxTM) was from Pfizer S.A., Argentina. Extracts containing *M. bacilliformis* protease were prepared according to Fernandez Lahore *et al.*[6]. All other reagents were AR grade.

Preparation of dye-Sepharose 4B

Reactive dyes were immobilised on Sepharose 4B as described by Stellwagen[7] with minor modifications: 1 g of Sepharose 4B was suspended in 2 ml of water and 50 mg of dye was added prior to the addition of 1 ml of 2 M NaCl. Then, 0.5 ml of 0.1 N NaOH was added whenever a dichlorotriazine dye was used, and the mixture was stirred gently for 4 h at room temperature. If a monochlorotriazine dye was used, 0.5 ml of 1 N NaOH was added and the mixture stirred for 16 h at 60° C. The mixture was then filtered and the matrix washed with water, 1 M NaCl, 2 M NH4Cl and water once again, until the filtrate was clear. The amount of immobilised dye was determined by acid hydrolysis followed by a spectrophotometric measurement as described by Burton et al.[8].

Acid protease purification screening experiments

One-ml bed volume (1 cm x 1.3 cm) columns were prepared, each having one out of 9 different dye-Sepharose matrix. Each column was first equilibrated with 5 ml of starting buffer and then loaded with 0.5 ml of acid protease solution in the same buffer. After a washing step with 5 ml of equilibrating buffer, the elution was performed with 4 ml of elution buffer. Samples from the washing and elution steps were monitored for milk clotting activity as described by Arima *et al.*[9]. Columns were regenerated by flushing 6 ml of 50 mM NaOH followed by 6 ml of water.
The equilibrating buffers assayed were: 50 mM sodium acetate, pH 4.1, 5.4 and 6.5. In all cases the elution was performed with a 50 mM sodium acetate buffer, pH 6.5, 2 M NaCl.

Analytical methods

Total protein was determined according to Bradford[10]. SDS-PAGE 12.5 % was carried out in a Hoefer SE200 Mighty Small mini-gel unit (Pharmacia Biotech, Uppsala, Sweden), by using the discontinuous method described by Laemmli[11].

RESULTS AND DISCUSSION

Table 1 shows results obtained when chymosin was chromatographed on the 9 immobilised triazinic dyes, at pHs 4.1, 5.4 and 6.5.
At pH 4.1, chymosin was fully retained by all the dyes except for Scarlet GA. On the other hand, the elution degree with 2 M NaCl was very different and evidenced a wide spectrum of binding affinity strengths. At pH 5.4, Scarlet GA did not retain chymosin at all while it was fully retained by all the other dyes, the same as had occurred at pH 4.1. However, the affinity binding strength was lower as judged by the higher percentages of eluted enzyme. At pH 6.5, in contrast, all assayed dyes except for Red F5B and Yellow FR were unable to retain significant amounts of chymosin. Results denote that the chromatographic behaviour of chymosin agrees with the general trend of decreased retention when pH is raised[12]. The behaviour of Red F5B, fully retaining chymosin at pH 6.5 and being eluted to a very high percentage with 2 M NaCl, indicates that Red F5B is a useful ligand for affinity purification of chymosin. Subramanian[5] purified chymosin from rennet with Cibacron Blue-Sepharose at pH 5.5. According to our results, Red F5B appears as an advantageous alternative to Cibacron Blue, as chymosin is fully retained at a higher pH (6.5) - thus making more selective the binding step - and then it is eluted by ion strength increase.
Table 2 shows results obtained when pure *M. miehei* acid protease was chromatographed on the same set of dyes. The behaviour of this enzyme, though following the same general trend as chymosin as regards pH, markedly differs in quantitative terms: at pH 4.1, Yellow HE-R, Orange HE-R and Red HE-7B did not bind M. miehei protease significantly, although fully binding chymosin. At pH 5.4, only Red F5B was able to bind a significant amount of this enzy-

TABLE I
Chromatographic behaviour of chymosin on 9 immobilised triazinic dyes

Dye	Dye density (μmol/ml)	pH 4.1		pH 5.4		pH 6.5	
		R[1]	E[2]	R	E	E	R
Yellow FR	3.6	100	13	95	71	50	49
Yellow HE-R	3.2	100	92	100	88	5	N/D
Orange HE-R	3.7	100	97	99	98	0	N/D
Scarlet GA	3.9	31	30	0	N/D	0	N/D
Red HE-3B	3.1	100	33	97	74	4	N/D
Red HE-7B	3.1	100	72	98	83	3	N/D
Red F5B	3.2	100	65	100	88	99	93
Blue HE-R	3.5	100	75	100	94	1	N/D
Cibacron Blue	3.8	100	73	97	54	8	N/D

[1] Percentage of the loaded enzyme activity not found in the column wash (R,retained).
[2] Percentage of the loaded enzyme activity eluted from the column by 2 M NaCl in the equilibrating buffer (E, eluted).
N/D: not determined.

TABLE II
Chromatographic behaviour of *Mucor miehei* acid protease on 9 immobilised triazinic dyes

Dye	pH 4.1		pH 5.4	
	Retained[1]	Eluted[2]	Retained	Eluted
Yellow FR	100	79	8	N/D
Yellow HE-R	4	N/D	2	N/D
Orange HE-R	27	25	12	12
Scarlet GA	0	N/D	0	N/D
Red HE-3B	90	76	6	N/D
Red HE-7B	5	N/D	4	N/D
Red F5B	100	89	82	78
Blue HE-R	98	94	0	N/D
Cibacron BlueF3G-A	100	78	1	N/D

[1] Percentage of the loaded enzyme activity not found in the column wash.
[2] Percentage of the loaded enzyme activity eluted from the column by 2 M NaCl in the equilibrating buffer.
N/D: not determined.

TABLE III
Chromatographic behaviour of *Mucor bacilliformis* acid protease on 9 immobilised triazinic dyes

Dye	pH 4.1		pH 5.4	
	Retained[1]	Eluted[2]	Retained	Eluted
Yellow FR	60	43	0	N/D
Yellow HE-R	82	77	3	N/D
Orange HE-R	82	49	10	N/D
Scarlet GA	0	N/D	0	N/D
Red HE-3B	99	95	11	N/D
Red HE-7B	54	34	0	N/D
Red F5B	89	68	5	N/D
Blue HE-R	60	44	1	N/D
Cibacron Blue F3G-A	76	67	2	N/D

[1] Percentage of the loaded enzyme activity not found in the column wash.

[2] Percentage of the loaded enzyme activity eluted from the column by 2 M NaCl in the equilibrating buffer.
N/D: not determined.

TABLE IV
Purification of an acid protease from a crude extract of *Mucor bacilliformis* solid-state culture by affinity chromatography with immibilised Red HE-3B

Fraction	Milk clotting activity (CU)	Total protein (mg)	Specific activity (CU/mg)	Yield (%)	Purification factor
Load	924	7.0	132	100	
Wash	50	5.2	9.6		
Elution	880	1.4	629	95.2	4.8

me while chymosin did so to 8 out of the 9 dyes assayed. At pH 6.5, no dye retained *M. miehei* protease.
These results indicate that Red F5B is also a potentially useful ligand for *M. miehei* protease purification at pH 5.4 while this dye is useful with chymosin up to pH 6.5.
Table 3 shows the results of the assay performed with a milk-clotting protease purified from an extract of a solid-state culture of *M. bacilliformis*. This protease has been proposed as a substitute for chymosin in the manufacture of cheese[13]. At pH 4.1, all dyes - except for Scarlet GA - bound over 50% protease while, at pH 5.4, only Orange HE-R and Red HE-3B bound just about 10% enzyme. Results suggest that Red HE-3B could be a useful ligand for affinity purification of *M. bacilliformis* acid protease. In order to check this possibility, a test with immobilised Red HE-3B at pH 4.1 was performed. When the clarified aqueous crude extract of the *M. bacilliformis* solid-state culture was set to pH 4.1 and loaded to the column, the protease failed to be retained, thus suggesting that some of the components of the crude extract could interfere with the binding of the enzyme to the immobilised dye. A similar interference was reported for ion-exchange purification of the M. bacilliformis protease[6] and for the affinity purification of other proteins[14]. However, after conditioning of the crude extract by size-exclusion chromatography on a PD-10 column equilibrated in 50 mM sodium acetate buffer, pH 4.1, the acid protease bound to the immobilised dye. Table 4 shows the purification record of the *M. bacilliformis* protease on the dye affinity column selected. Purified protease yielded a single band at 31 kDa in SDS-PAGE (not shown) thus evidencing the usefulness of the method proposed.
Results obtained in this work show that chymosin displays a higher affinity for triazinic dyes than *M. miehei* and *M. bacilliformis* proteases, thus suggesting that, despite the high structural and functional homology, there are sequence and/or conformational differences responsible for the dissimilar affinity to dyes between fungal acid proteases and chymosin.

CONCLUSIONS

Usefulness of the screening of immobilised triazinic dyes to find ligands for affinity purification of acid proteases from different origin was demonstrated.
Red F5B was identified as the best ligand for chymosin and *Mucor miehei* acid protease purification while Red HE-3B allowed purification of *Mucor bacilliformis* acid protease from crude extracts.

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BIBLIOGRAPHY

[1]. Boyer, P.M. and Tsu, J.T. (1993) *Adv. Biochem. Eng. & Biotechnol.* **49**:1-44.
[2]. Ibrahim-Granet, O. and Bertrand, O. (1996) *J. Chromatogr.B* **684**:239-263.
[3]. Madhusudan,M. and Vijay, M. (1992) *Prot. Eng.* **5**:399-404.
[4]. Scopes, R.K. (1987) *Analyt. Biochem.* **165**:235-246.
[5]. Subramanian, S. (1987) *Prep. Biochem.* **17**:297-312.
[6]. Fernández Lahore, H.M., Fraile, E.R. and Cascone, O. (1998) *J. Biotechnol.* **62**:83-93.
[7]. Stellwagen, E. (1990) In: *Methods in Enzymology*, Academic Press, vol. 182, pp. 343-357, London.
[8]. Burton, S.J., Stead, C.V. and Lowe, C.R. (1988) *J. Chromatogr.* **435**:201-216.
[9]. Arima, K., Yu, J. and Iwasaki, S. (1970) In: *Methods in Enzymology*, Academic Press, vol. 9, pp. 446-459, New York.
[10]. Bradford, M. (1976) *Analyt. Biochem.* **72**:248-254.
[11]. Laemmli, U.K. (1970) *Nature* **227**:680-685.
[12]. Angal, S. and Dean, P.D.G. (1978) *FEBS Lett.* **96**:346-350.
[13] .Areces, L.B., Biscoglio de Jiménez Bonino, M.J., Parry, M.A.A., Fraile, E.R., Fernández, H.M. and Cascone, O. (1992) *Appl. Biochem. Biotechnol.* **37**:283-294.
[14]. Rozie, H., Somers, W., Bonte, A., Visser, J., vant'Riet, K. and Rombouts, F.M. (1988) *Biotechnol. Appl. Biochem.* **10**:346-358.