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Purification of soybean peroxidase (*Glycine max*) by metal affinity partitioning in aqueous two-phase systems

Maria Estela da Silva, Telma Teixeira Franco*

Biochemical Engineering Laboratory, Chemical Engineering, State University of Campinas (UNICAMP), P.O. Box 6066, Campinas, SP 13081-970, Brazil

Abstract

Combining two concepts in downstream processing, this work investigates the partitioning of a crude soybean peroxidase (*Glycine max*) in an aqueous two-phase system by metal affinity. A liquid–liquid extraction process using metal ligands was developed in two steps with the aim of purifying the enzyme peroxidase. PEG 4000 was activated using thionyl chloride, covalently linked to iminodiacetic acid (IDA), and the specific metal ligand Cu^{2+} was attached to the PEG 4000–IDA. In the first step, the system was composed of 14% (w/w) PEG 4000–IDA–Cu^{2+} and 8% (w/w) Na_2SO_4, and the peroxidase partitioned mainly to the top phase ($K = 24$). In the second step, a system formed by 14% PEG 4000 and 10% phosphate was used to revert the value of the partition coefficient of peroxidase to the bottom salt-rich phase ($K = 0.05$), thereby achieving a recovery of 64% of the purified enzyme.

Keywords: *Glycine max*; Metal affinity partitioning; Aqueous two-phase systems; Soybean peroxidase; Enzymes

1. Introduction

Partitioning in an aqueous two-phase system (ATPS) is a selective method used for biomolecule purification [1,2]. The tendency for two different aqueous polymers (polyethylene glycol and dextran) or a polymer and a lyotropic salt to separate into two distinct phases in a common solvent has been recognized since the end of the last century. For example, a mixture of sodium sulphate and polyethylene glycol dissolved in water is turbid above certain concentrations and the two phases are in equilibrium. The lighter phase is enriched in PEG while the heavier is enriched in salt. Metal ions are potentially useful to selectively extract, by affinity partitioning, proteins which have histidine, cysteine and tryptophan on their surface [3–8], including proteins of clinical and industrial interest [9], in a simple and rapid procedure [10]. The iminodiacetic acid (IDA) chelator is coupled by covalent binding to one of the phase-forming polymers; therefore, its partitioning occurs either in the top or in the bottom phase and the metal ions are loaded into the chelate polymer. If the target protein to be isolated has an affinity for the metal ligand, the formation of the metal–protein complex will alter the partitioning of the protein [11–13]. Metal affinity partitioning in ATPS offers a fast and selective alternative for extracting enzymes with good applicability to scale-up and can replace

*Corresponding author. Tel.: +55-19-788-3966; fax: +55-19-788-3965.

E-mail address: franco@feq.unicamp.br (T. Teixeira Franco)

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some of the chromatographic steps in downstream processing.

The main advantages of using metals as ligands are: (1) they can be recycled many times with insignificant loss in performance, (2) high metal loading and therefore high protein capacities can be attained, (3) product elution and ligand regeneration are achieved with relative ease, (4) the cost of the metals used is low [14], (5) they are stable under a wide range of solvent conditions and temperatures, (6) their interactions with target molecules are reasonably specific and (7) these interactions are reversible under mild conditions [12].

Peroxidase (EC 1.11.1.7) is the enzyme most frequently used in the manufacture of enzyme immunoassay kits [15] and medical diagnosis kits [16]. It is used in the enzymatic determination of glucose content, especially for people suffering from diabetes [17]. This enzyme can also be employed in biosensors, the transformation of drugs, the production of chemicals, the degradation of aromatic compounds, environmental control [18] and in processes for making a wide variety of chemical intermediates, as well as formaldehyde-free phenolic resins [19]. Another important class of materials produced by peroxidase-catalysed reactions in organic solvents is the polyaromatic amines and polyphenols [20].

Several methods for peroxidase extraction and purification have been described in the literature. A 628-fold purified soybean peroxidase was obtained by using a sequence of different procedures consisting of ammonium sulphate fractionation, gel filtration chromatography, ion-exchange chromatography, affinity chromatography and hydrophobic chromatography, achieving a 4% yield [21]. Another extraction procedure was developed for purifying soybean peroxidase in a temperature-induced ATPS system formed of Triton X-45, Triton X-100 and sodium acetate at pH 5.5. Affinity chromatography was used in a second step, increasing the purification factor 41-fold and a 28% yield of peroxidase [22]. Peroxidase of hulls of *Glycine max* has been purified 57-fold after a four-step process achieving a 16.4% yield of the enzyme [23].

This article reports the feasibility of the extraction and purification of soybean peroxidase, *Glycine max*, by metal affinity partitioning in ATPS.

2. Experimental

2.1. Chemicals

PEG 4000 was purchased from Synth (Buchs, Switzerland), thionyl chloride from Aldrich (Steinheim, Germany), iminodiacetic acid (IDA), Coomassie blue G250, soya peroxidase and copper sulphate from Sigma (St. Louis, MO, USA), and sodium sulphate, sodium bicarbonate and hydrogen peroxide from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Crude enzyme extract

Crude extract was prepared by crushing and drying ordinary seeds of *Glycine max* PL1, kindly donated by Instituto Agronômico de Campinas, São Paulo, Brazil. One gram of defatted soybean flakes was mixed in 10 m*M* phosphate buffer, pH 8.0, for 2 h at 4°C (1:25, w/w) and then centrifuged at 5000 *g* for 30 min. The supernatant was maintained at 4°C.

2.3. Phase diagrams

The binodial curves of the ATPS composed of PEG 4000–sodium sulphate and PEG 4000–potassium phosphate were constructed according to Refs. [1,24]. Five grams of a concentrated stock solution (40% w/w) of potassium phosphate (or 20% w/w sodium sulphate) were stirred constantly and a solution of 50% (w/w) PEG 4000 was titrated. An homogeneous mixture was initially obtained, and after a given amount of PEG had been added, one additional drop caused turbidity and a two-phase system arose. Deionized water was added drop by drop until the mixture lost its turbidity. More PEG solution was then added and a two-phase system was again obtained. The full procedure was repeated in triplicate until a series of compositions close to the binodial was obtained, and the concentration of phosphate or sulphate plotted versus PEG gave a binodial curve for the system. As a convention, the concentration of the compound whose distribution favours the bottom phase is plotted as the abscissa, and the concentration of the polymer, which distributes into the top phase, is plotted as the ordinate.

The tie lines were constructed according to Ref. [25]. Forty-five grams of ATPS were prepared by weighing stock polymer solutions and dry salts in a 50 ml equilibrium cell. The systems were mixed for 2 min and then separated at room temperature. Approximately 500 mg of phase were weighed in a glass tube and two volumes of water were added. The solutions were shell-frozen in a mixture of dry ice and acetone. A lyophilizer was used to sublimate the water in vacuum at 13.3 Pa for 24 h, after which the tubes were again weighed. The sample tubes were placed on the surface of a hot plate at approximately 450°C for 5 days. The PEG was oxidised and volatilised while the salt remained as a white ash. The tubes were repeatedly weighed until the mass was constant.

2.4. Synthesis of PEG–IDA–Cu^{2+}

The PEG–IDA–Cu^{2+} were synthesised according to Ref. [12].

2.4.1. Activation of PEG 4000 with thionyl chloride

(**I**) Solid PEG 4000 (30 g) was dried in vacuum at 55°C for 5 h. Then 5.5 ml of thionyl chloride was added and the melt was reheated at 65°C for 5 h. Excess thionyl chloride was removed in vacuum and the melt was dried (PEG-Cl).

2.4.1.1. Attachment of IDA to PEG-Cl. Twenty-five grams of compound **I** (PEG-Cl) were dissolved in 100 ml of water and 15 g IDA and 10 g potassium carbonate were added and mixed and refluxed for 48 h. Ten grams of sodium sulphate were added to the hot reaction mixture and allowed to cool and to separate into two phases. The PEG (top) phase was retained and diluted to 120 ml with water. Solid impurities were removed by filtration through a 0.45 μm membrane. The reagents were removed by diafiltration with 1% sodium bicarbonate solution and extensively with water for 48 h and finally lyophilized (**II**).

2.4.1.2. Attachment of Cu^{2+} to PEG–IDA. Five grams of compound **II** and 3 g of copper sulphate were dissolved in 5 ml water and allowed to equilibrate for 24 h. The solution was diluted to 20 ml, filtered through a 0.45 μm filter and then ultrafiltered with 1000 ml of water to remove the excess copper. The content (**III**) was lyophilised and used to prepare aqueous two-phase systems.

2.5. Two-phase system preparation

Aqueous two-phase systems were prepared from stock solutions of PEG 4000 (50%, w/w), Na_2SO_4 (20%, w/w) and potassium phosphate solution with K_2HPO_4–KH_2PO_4 (40%, w/w) with a molar ratio of 0.64, according to Refs. [26,27]. Solid PEG–IDA–Cu^{2+} was used to prepare the affinity ATPS. In the first step, the compounds were mixed to form 8.0 g of a system having a final concentration of 10% PEG–IDA–Cu^{2+} (compound **III**), 4% PEG 4000 and 8% sodium sulphate. The pH of the system without the ligand was adjusted to be equal to the system with PEG–IDA–Cu^{2+}. In the second step, the bottom phase of the PEG–IDA–Cu^{2+}–sulphate system was discarded and replaced with a fresh phosphate phase, pH 6.0. The composition of the system (6.0 g total mass) was 14% PEG 4000–IDA–Cu^{2+} and 10% K_2HPO_4–KH_2PO_4, pH 6.0. The system was mixed and centrifuged. In the recycling of PEG–IDA–Cu^{2+}, the top phase of the PEG–IDA–Cu^{2+}–phosphate system was collected and washed 10 times with water by diafiltration with a YM1 membrane from Amicon (Beverly, MA, USA) and concentrated to reuse in other purification cycles.

2.6. Partition coefficient and peroxidase activity

Three hundred microliters of enzyme extract were mixed with the PEG–IDA–Cu^{2+}–sulphate system using a Vortex for 1 min. Phase separation was achieved by centrifugation for 3 min at 3000 *g*, and the phases were carefully separated and the interface of each tube discharged. A known volume of each phase was transferred and peroxidase activity was assayed. The assay mixture contained 3.0 ml of 100 m*M* potassium phosphate buffer, pH 7.0, 50 μl of guaiacol and 50 μl sample aliquots. The reaction was started by the addition of 40 μl of 8 m*M* hydrogen peroxide, and the absorbance at 436 nm was recorded within 0–5 min intervals and the enzyme

activity was calculated [28]. The partition coefficient of the enzyme, K_E, is calculated as the ratio of enzyme activity in the top phase to that in the bottom phase at room temperature:

$$K_E = \frac{[\text{enzyme}]_{\text{top phase}}}{[\text{enzyme}]_{\text{bottom phase}}}$$

2.7. Protein assay

The main contaminant protein concentration was determined by the dye-binding technique [29]. Fifty to 100 μl of the top phase were transferred from each prepared system to a cuvette containing 2.4 ml of water and 1.0 ml of Coomassie blue G250 (Sigma) in 2.2% hydrochloric acid, and the OD_{595} was measured against a blank which had 50–100 μl of a top phase of a system prepared in the same way without any sample. The blanks were prepared to correct the interference of the phase components. The procedure was repeated for the bottom phase of each system. A bovine serum albumin (BSA) standard curve was used to calculate protein concentration. The partitioning of the main contaminant protein, K_P, was calculated as the ratio of protein concentration in the top phase to that in the bottom phase at room temperature:

$$K_P = \frac{[\text{protein}]_{\text{top phase}}}{[\text{protein}]_{\text{bottom phase}}}$$

2.8. Electrophoresis

SDS electrophoresis (SDS–PAGE) was carried out in 12% homogeneous gel [30]. The gels were stained with Bio-Rad silver (Hercules, CA, USA). The molecular mass (M_r) markers consisted of phosphorilase b (94 000), BSA (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and α-lactalbumin (14 400) available as a standard kit (Amersham-Pharmacia Biotech, Uppsala, Sweden).

2.9. Specific peroxidase activity (SA_{perox})

This was defined as the ratio of enzyme activity (U/ml) to total protein concentration (mg/ml) and expressed in U/mg of protein:

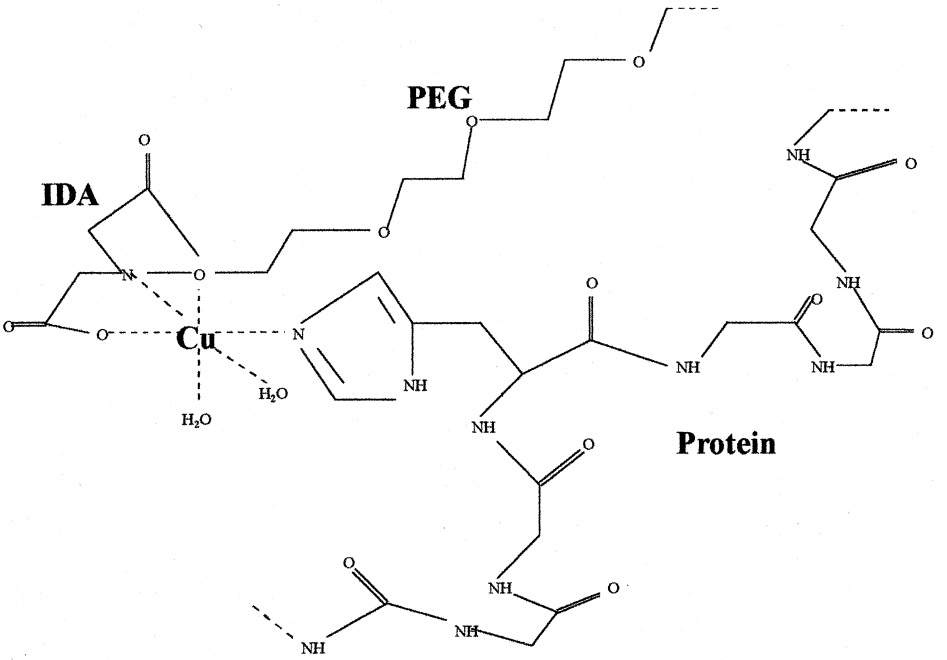

Fig. 1. PEG–IDA–Cu^{2+}–protein complex.

Table 1
Partitioning of three different peroxidases bound to L-histidines in ATPS

System (%)			Lactoperoxidase			Horseradish peroxidase		Soybean peroxidase		
PEG	Phosphate	PEG–IDA–Cu^{2+}	K_E	K_P	R (%)	K_E	R (%)	K_E	K_P	R (%)
13	9	–	0.16	0.06	86	5.42	70	2.03	0.03	90
12	9	1	0.17	0.05	73	150	54	3.74	0.04	100
8	9	5	0.18	0.93	95	180	55	37	2.99	75
9	9	10	3.31	1.10	95	–	–	–	–	–

$$SA_{perox} = \frac{\text{enzyme activity}}{\text{protein concentration}} \quad (1)$$

2.10. Purification factor (PF)

The PF is defined as the ratio of the specific peroxidase activity after a purification step to the initial specific peroxidase activity (from the crude enzyme extract or from a previous purification step):

$$PF = \frac{SA_{perox} \text{ in the collected phase}}{\text{initial } SA_{perox}} \quad (2)$$

2.11. Recovery (R)

This is defined as the ratio of the enzyme activity of the aqueous phase after partitioning to the total enzyme activity added to the system:

$$R(\%) = \frac{\text{enzyme activity of the phase}}{\text{total enzyme activity added to the system}} \quad (3)$$

2.12. Selectivity (S)

This is defined as the ratio of the partition coefficient of the enzyme, $K_{E,}$ to the partition coefficient of the protein, K_P:

$$S = \frac{K_E}{K_P} \quad (4)$$

3. Results and discussion

In order to isolate peroxidase from the crude soybean enzyme extract, a metal affinity system was investigated. Initially, the affinity polymer was prepared by covalent binding of IDA to the activated PEG 4000-Cl. Then the metal ion complex was prepared by dissolving copper sulphate salt in the PEG–IDA stock solution. The metal-loaded chelate PEG interacts with the accessible residues of histidine, cysteine and tryptophan available on the protein surface (Fig. 1) [31].

Preliminary experiments had shown that the soybean oil from the seeds inhibited binding of per-

Table 2
Liquid–liquid extraction process of soybean peroxidase[a]

System	K_E	K_P	R (%)	SA	PF	S
A: 14% PEG 4000 8% Na_2SO_4	1	0.29	78 (total phase)	23	1.3	3.5
B: 4% PEG 4000 + 10% PEG–IDA–Cu^{2+} + 8% Na_2SO_4	24	1.7	106 (top phase)	35	1.5	14
C: 4% PEG 4000 + 10% PEG–IDA–Cu^{2+} + 10% phosphate	0.05	76.0	64 (bottom phase)	3333	145	$7 \cdot 10^{-4}$
Recycle 4% PEG 4000 8% Na_2SO_4 10% PEG–IDA–Cu^{2+}	22.8	1.4	85 (top phase)	65	2.83	16

[a] Crude extract: peroxidase activity 13 U/ml, protein concentration 0.58 mg/ml, specific activity 23 U/mg.

oxidase to PEG–IDA–Cu^{2+}; therefore, the crude enzyme extract used was prepared from oil-free crushed seeds. It was observed that the crude extract of soybean contained proteins of several different molecular masses in the range from M_r 100 000 to 10 000.

The performance of the prepared metal–chelate PEG was tested by attaching L-histidine to three different peroxidases: horseradish peroxidase, soybean peroxidase and lactoperoxidase. Table 1 shows that the partition coefficient of the horseradish peroxidase bound to L-histidine increased more (33-fold) than the others. The literature reports the use of metal affinity chromatography to recover horseradish peroxidase also bound to L-histidine [32].

When the enzyme extract was partitioned in 14% PEG 4000–8% sodium sulphate (system A) without metal ligand, the partition coefficient of peroxidase, K_E, was 1.0, but the K_P of the main contaminant proteins was 0.29, and 78% of the peroxidase activity was recovered in the bottom phase (Table 2). By replacing 10% of the plain PEG 4000 by a corresponding amount of PEG–IDA–Cu^{2+} (system B), the peroxidase K_E value increased to 24 (a 24-fold increase) and the K_P value increased approximately six-fold ($K = 1.7$). Peroxidase yield in the top phase was above 100%, indicating the preference for peroxidase in the IDA–Cu^{2+}-rich phase. The amount of PEG–IDA–Cu^{2+} added to the system corresponded to about 71% of the total PEG content.

In order to improve the purification of the peroxidase, the bottom sulphate-rich phase of system B was discarded and a new fresh phosphate phase was prepared. Binodial curves and tie lines of PEG 4000–sodium sulphate and of PEG 4000–phosphate systems were then studied in order to calculate how much of each component should be added to the system. The binodial curve of the PEG 4000–sulphate shows that the top phase of system B contained 31% PEG 4000 and 2.2% sulphate and the bottom phase contained 1% PEG 4000 and 13% sodium sulphate (Fig. 2). It was observed that K_E was greatly changed by disrupting the interaction between the copper complex and the soy peroxidase. Therefore, the top PEG–IDA–Cu^{2+} phase was mixed with a stock phosphate solution to give a final composition of 14% PEG–IDA–Cu^{2+} and 10% phosphate (system C). As can be seen from the

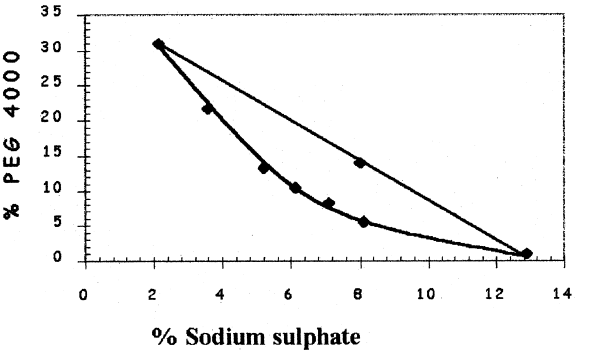

Fig. 2. Binodial curve and tie line for system B, 14% PEG 4000 + 8% sodium sulphate + water, 25°C.

binodial curve and tie line (Fig. 3), the new top PEG-rich phase contained 30% PEG 4000 and 3% phosphate and the bottom phosphate-rich phase contained 2% PEG 4000 and 15% phosphate. However, as the top PEG-rich phase of the PEG 4000–sulphate system was not previously ultrafiltered, the new PEG 4000–IDA–Cu^{2+}–phosphate system still contained a low concentration of sulphate ion (<2%). It was assumed that the binodial curve of the PEG 4000–IDA–Cu^{2+}–sulphate system was similar to that of the PEG 4000–sulphate system.

Our studies showed that when the crude enzyme extract was partitioned in a 14% PEG 4000 and 10% phosphate system, pH 6.0, the peroxidase concentration was high in the salt-rich phase and the K_E

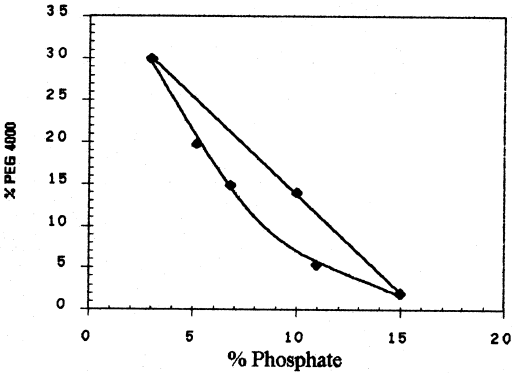

Fig. 3. Binodial curve and tie line for system C, 14% PEG 4000 + 10% phosphate (K_2HPO_4–KH_2PO_4, molar ratio 0.6) + water, 25°C.

value was 0.05. Therefore, the peroxidase, which had previously been collected in the top-affinity phase of the PEG–IDA–Cu^{2+}–sulphate system (system B), partitioned in the salt–phosphate rich phase. A 64% recovery of the total peroxidase initially added to system B was collected in the bottom phosphate-rich phase of system C in the second-step partitioning. The purification factor achieved was 145 and the selectivity of the system was $7 \cdot 10^{-4}$, indicating that K_P was 1520 times higher than K_E. The peroxidase-specific activity in the phosphate phase was 3333 U/mg of protein. Fig. 4 shows a clear electrophoretic band of a protein of approximately M_r 30 000 in the material collected from the top phase of system B (lane d), corresponding to a band also observed for the standard peroxidase (lane c). The gel shows other bands corresponding to proteins with lower molecular masses, which appear in the same material. However, a comparison between the innumerable electrophoretic bands of the commercial standard peroxidase (lane c) and those found in the material collected from the affinity step clearly shows the efficiency of the process developed. The top phase of the metal affinity system B was reused for peroxidase extraction, and it was observed that K_E and K_P values for the enzyme recovery and selectivity of the system were not significantly changed.

4. Conclusions

A two-step extractive liquid–liquid process was developed with the objective of purifying the enzyme peroxidase from a crude extract of *Glycine max*.

In the first step of the process developed, ATPS was composed of 4% PEG 4000, 10% PEG–IDA–Cu^{2+} and 8% Na_2SO_4 and the value of the partition coefficient of peroxidase, K_E, was 24, which was a 24-fold increase when compared to the initial value in an ATPS without the metal ligand (K was 1.0). The bottom salt-rich phase of this system was discarded and a new phosphate phase was mixed with the top PEG-rich phase. Therefore, in the second step of the purification process, a system formed of 4% PEG 4000, 10% PEG–IDA–Cu^{2+} and 10% phosphate was used to revert the value of the partition coefficient of the peroxidase ($K = 0.05$), thereby achieving a 64% yield of the enzyme. The purification factor of the peroxidase collected in the phosphate phase was 145. Electrophoresis shows that three main large bands appear in the material collected from the affinity phase, indicating that this material is purer than the commercial standard soybean peroxidase. The experiments also show the possibility of reusing PEG–IDA–Cu^{2+} in two-phase systems.

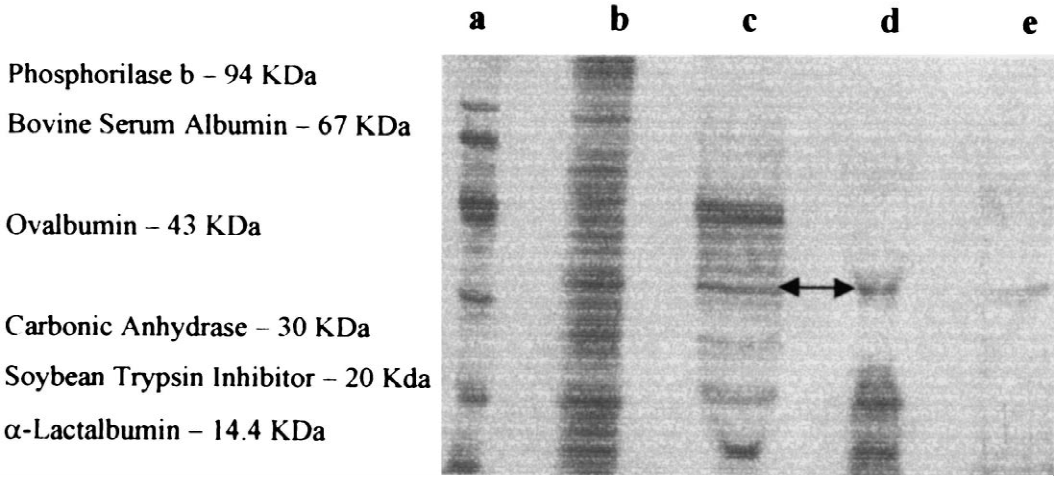

Fig. 4. SDS–PAGE gel electrophoresis of crude enzyme extract of soybean purified by metal affinity partitioning. Lane a, molecular mass (M_r) markers consisting of phosphorilase b (94 000), BSA (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and α-lactalbumin (14 400); lane b, crude peroxidase extract; lane c, soybean standard peroxidase; lane d, top phase of system B; lane e, bottom phase of system B.

5. Nomenclature

ATPS	aqueous two-phase system
BSA	bovine serum albumin
PEG	polyethyleneglycol
IDA	iminodiacetic acid
K	partition coefficient
PF	purification factor
AS	specific activity
R	recovery
S	selectivity

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